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Influence of the content in dietary polyunsaturated fatty acids on lipid metabolisms and immune responses of common carp (Cyprinus carpio)

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INFLUENCE OF THE CONTENT IN DIETARY POLYUNSATURATED FATTY ACIDS ON LIPID METABOLISM AND IMMUNE RESPONSES IN COMMON CARP (*Cyprinus carpio*) – *IN VIVO* AND *IN VITRO* APPROACHES

A dissertation submitted by

NGUYEN THI MAI

in partial fulfilment of requirements

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Influence of the content in dietary polyunsaturated fatty acids on lipid metabolisms and immune responses of common carp (*Cyprinus carpio*)

in vivo and *in vitro* approaches

By NGUYEN Thi Mai

SUMMARY

Context: Common carp *Cyprinus carpio* is an important aquaculture species; it is the most cultured fish for human food consumption. As many other freshwater fish species, common carp is able to biosynthesize the long chain polyunsaturated fatty acids (LC-PUFAs) from PUFA precursors by a series of elongation and desaturation reactions. LC-PUFAs play an important role in fish immune system, and their imbalance or inadequate supply could lead to negative effects on fish health. LC-PUFAs released from cell membrane phospholipids participate in the metabolism of some molecules involved in the inflammatory processes. The eicosanoids including prostaglandins and leukotriene (produced from arachidonic acid, ARA and eicosapentaenoic acid, EPA) are among the main pro-inflammatory mediators; while lipoxins (synthesized from arachidonic acid, ARA) or resolvins from the n-3 LC-PUFAs such as DHA, act as anti-inflammatory factors. However, the information on the influence of LC-PUFA amounts on fish immune system via the pro- and anti-inflammatory responses in fish in general, and in common carp in particular, is still limited. In this context, the current thesis was conducted to determine the influence of dietary fatty acids (FA) amounts from various plant oil sources on (1) growth performance, feed conversion rate, and survival; (2) FA composition; (3) immune status and (4) pro and anti-inflammatory responses in common carp.

Research strategy and methodology: Four experiments were carried out during this thesis. The first experiment was designed using six oil sources including cod liver oil (CLO), linseed oil (LO), sesame oil (SO), sunflower oil (SFO) and two blends of these plant oils – SLO (SO + LO, *v:v, 1:1*) and SSFO (SO + SFO, *v:v, 1:1*) to determine the digestibility of candidate plant oils and their influence on fish growth and FA composition in common carp. The second experiment was then carried out using three dietary lipid sources (CLO, LO and SFO) in combination with an immunostimulant (β-glucan) to assess the immune status in common carp and their immunocompetence. To determine the influence of dietary FA composition on the immune responses in cell model, the third experiment was conducted combining *in vivo* and *in vitro* approaches during which head kidney leucocytes (HKL) and peripheral blood mononuclear cells (PBMC) were isolated from common carp fed with different dietary lipid sources (CLO, SO, LO and SLO). The cells were then exposed to *E.coli* LPS. The last experiment was performed using the same lipid sources as in the third experiment; moreover, two additional LC-PUFA-supplemented diets (LO + ARA, LOA and SO + DHA, SOD) were tested. This experiment aimed to assess the effects of dietary plant oils enriched in n-3 (linseed oil) or n-6 (sesame oil), or supplemented with ARA or DHA on the pro and anti-inflammatory responses in HKL isolated from fish fed different oils and submitted *in vitro* to a LPS stimulation.

Results: The tested lipid sources did not influence the fish growth and survival but a mixture of plant oils (SLO) induced a higher feed conversion rate compared to fish oil-fed group. FA profiles in fish muscle and liver were modified by the oil sources and reflected the dietary FA composition. Fish were able to biosynthesize LC-PUFAs from PUFA precursors conducting to high level of EPA (from ALA) in LO-fed fish compared to SFO and SO-fed ones or high level of ARA (from LA) in fish fed SO and SFO-based diets compared to other experimental groups even if these LC-PUFAs were totally absent in plant oil-based diets. The mixture of SO and LO (SLO diet) induced the positive effect via balanced LC-PUFAs in fish compared to their pure plant oils. Lysozyme activity in fish fed SFO+ did not differ from SFO group; however, the overall immune status of plant oil-fed fish reared under normal conditions or challenged intraperitoneally with *A.hydrophyla* (at dose of 5×10^8 CFU) did not significantly differ from the one of fish fed cod liver oil. Besides, several genes involved in eicosanoid metabolism were up-regulated in SFO-fed fish reared under the normal conditions. A dietary SLO induced the highest levels of peroxidase activity and expression of gene involved in eicosanoid metabolism processes (*pge2*). The gene expressions of cytokines or other mediators involved in pro- and anti-inflammatory responses were dependent on time and LPS-dose, and generally, these genes were up-regulated in early stage of LPS exposure. HKLs from fish fed the SLO diet which is more balanced in PUFA precursors, or vegetable diets supplemented with ARA (LOA) or DHA (SOD), exhibited the efficient regulation of acute inflammatory processes compared to CLO leukocytes.

Conclusion: Common carp are able to use the plant-derived oils without any negative effect on growth, feed conversion rate and survival. Fish fed ALA-enriched diet have exhibited the EPA level higher than other plant oil-fed groups while the highest value of ARA levels was found in LA-enriched ones. The blend of terrestrial vegetable oils or LC-PUFA supplementation in plant oil-based diets increased the immune responses when compared with those in fish fed pure plant oils and comparable to those observed in fish oil fed fish, especially in respect to pro- and anti-inflammatory processes. A combination of *in vivo* and *in vitro* approaches help to better understand the influence of lipid sources on the immune system of common carp.

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List of abbreviations

ACH50	:	Alternative complement activity	**LOX**	:	Lipoxygenase
ADC	:	Apparent digestibility coefficient	**LPS**	:	Lipopolysaccharide
ALA	:	α-linolenic acid	**LX**	:	Lipoxin
ARA	:	Arachidonic acid	**MALTS**	:	Mucosal-associated lymphoid tissues
CF	:	Crude fat	**MCH**	:	Major histocompatibility complex
CLO	:	Cod liver oil	**MUFA**	:	Monounsaturated fatty acid
COX	:	Cyclooxygenase	**NF-KB**	:	Nuclear factor kappa B
CP	:	Crude protein	**NO**	:	Nitrite oxide
CXC	:	Chemokine	**OA**	:	Oleic acid
DHA	:	Docosahexaenoic acid	**PAMP**	:	Pathogen associated molecular pattern
DM	:	Dry matter	**PBMC**	:	Peripheral blood mononuclear cells
DWG	:	Daily weight gain	**PG**	:	Prostaglandin
EPA	:	Eicosapentaenoic acid	**PL**	:	Phospholipid
FA	:	Fatty acid	**PLA**	:	Phospholipase
FAO	:	Food & Agriculture Organisation	**PUFA**	:	Polyunsaturated fatty acid
FBW	:	Final body weight	**RaRBC**	:	Rabbit red blood cells
FCR	:	Feed conversion rate	**Rv**	:	Resolvin
FO	:	Fish oil	**SFA**	:	Saturated fatty acid
GC	:	Gas chromatography	**SFO**	:	Sunflower oil
GE	:	Gross energy	**SFPC**	:	Soluble fish protein concentrate
GMO	:	Genetically modified organisms	**SGR**	:	Specific growth rate
HKL	:	Head kidney leukocytes	**SLO**	:	Sesame oil + linseed oil
IBW	:	Initial body weight	**SO**	:	Sesame oil
IFN	:	Interferon	**SOD**	:	Sesame oil + docosahexaenoic acid
Ig	:	Immunoglobulin	**SSFO**	:	Sunflower oil + sesame oil
IL	:	Interleukin	**TL**	:	Leukotriene
LA	:	Linoleic acid	**TLR**	:	Toll-like receptor
LC-PUFA	:	Long chain polyunsaturated fatty acid	**TNF**	:	Tumour necrosis factor
LO	:	Linseed oil	**TX**	:	Thromboxane
LOA	:	Linseed oil + Arachidonic acid	**WG**	:	Weight gain

Chapter 2

Thesis objectives and outlines

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The current thesis aimed to investigate the capacity of common carp to efficiently use dietary plant-derived oils in substitution to fish oil-based diets and to determine to what extent these alternative dietary plant oil sources affect growth performance, tissue fatty acid composition and immune status of common carp.

Firstly, we hypothesized that common carp, known to efficiently elongate and desaturate PUFAs into LC-PUFAs, could valorise plant oil-based diets, assuming that the FA compositions of pure or blended plant oils are adequate (hypothesis 1). In order to verify such hypothesis, we first determined the digestibility of different plant oils in comparison with fish oil-based diet, and then we evaluated the effects of these plant oil-based diets on fish growth, survival, feed efficiency, and tissue FA compositions. The results of this study are presented in chapter 4 entitled: "Digestibility of different plant-derived oils and influence of their combination on fatty acid composition of liver and muscle in juvenile common carp (*Cyprinus carpio*)".

On the basis of the results obtained in chapter 4, we hypothesized that the best plant oil-based diets could maintain a similar immunocompetence as the one usually observed in common carp fed fish oil-based diet (hypothesis 2). In order to verify this second hypothesis, we investigated the influence of LC-PUFA contents accumulated in common carp fed different lipid sources combined with β-glucan on the fish immune responses by an *in vivo* experiment and a bacterial challenge. The results of this research are shown in chapter 5 entitled: "Growth performance and immune status in common carp (*Cyprinus carpio*) as affected by plant oil-based diets complemented with β-glucan".

Results from chapters 4 and 5 suggested that membrane phospholipid LC-PUFA compositions and amounts of immune cell, known to participate in the eicosanoid productions, are modified by dietary FA profiles (hypothesis 3). In order to verify this hypothesis, we evaluated the molecular and physiological mechanisms involved in the interaction between lipid nutrition and immune responses in leukocytes isolated from common carp fed different oil sources and submitted *in vitro* to LPS stimulation. The results of this research are shown in chapter 6 entitled: "A combined *in vivo* and *in vitro* approach to evaluate the influence of linseed oil or sesame oil and their combination on innate immune competence and eicosanoid metabolism processes in common carp (*Cyprinus carpio*)".

Through the results obtained in chapter 6, we hypothesized that membrane phospholipid LC-PUFA compositions and amounts of immune cell, known to act as precursors of pro- and anti-inflammatory lipid mediator productions, are modified by dietary FA profiles, assuming that the supplementation of LC-PUFAs to dietary pure plant oils imbalanced in PUFA profiles could maintain a similar inflammatory regulations in immune cells of common carp as fish oil (hypothesis 4). In order to verify this hypothesis, we evaluated the influences of free LC-PUFAs supplemented in pure plant oil-based diets on the pro- and anti-inflammatory processes in common carp HKLs exposed to LPS. The results of this research are shown in chapter 7 entitled: "Pro- and anti-inflammatory responses of common carp *Cyprinus carpio* head kidney leukocytes to *E.coli* LPS as modified by different dietary plant oils".

A general discussion is provided in chapter 8 while chapter 9 provides a general conclusion and suggests some perspectives for future research on the interactions between fish nutrition, replacement of fish oil by plant oil in fish diets and impacts on fish immune status.

Chapter 3

Methodology

In this section, we briefly describe and explain the design of each experiment of the current study. Further, the general descriptions of the data analyses are presented.

Experimental design

This thesis consists of four experiments as follows:

Experiment 1: Digestibility of different plant-derived oils and influence of their combination on fatty acid composition of liver and muscle in juvenile common carp (Cyprinus carpio)

In order to verify hypothesis 1, the current experiment aimed at determining the digestibility of different plant oils in comparison to fish oil-based diet, and at evaluating the effects of these plant oil-based diets on fish growth, survival, feed efficiency, and tissue FA compositions.

In terms of diet formulations, six experimental diets were formulated using cod liver oil (CLO), linseed oil (LO), sunflower oil (SFO), sesame oil (SO) and two blends of linseed oil and sesame oil (SLO, *v:v 1:1*) or sesame oil and sunflower oil (SSFO, *v:v 1:1*). These oil sources were chosen basing on their natural FA compositions. CLO, enriched and balanced in LC-PUFAs, was used as a control diet. LO is rich in α-linolenic acid (ALA) while SO and SFO enrich in linoleic acid (LA). In this experiment, two plant oil mixture diets were formulated to provide different combinations of plant oils for fish diet. Fish meal was completely replaced by terrestrial plant and animal by-products.

This experiment consisted of two separate trials. Firstly, a digestibility trial of 14 days was conducted to determine the digestibility of four lipid sources (CLO, LO, SFO and SO) in common carp. Secondly, a growth trial of 96 days was performed to evaluate the growth performance, feed efficiency, survival, and tissue FA compositions in experimental fish.

Initial body weight (IBW), final body weight (FBW), feed intake, initial and final total numbers of fish were recorded to calculate the specific growth rate (SGR), feed efficiency (FE), and survival. Besides, fish muscle and liver were collected at the end of the nutritional trial to analyze the FA compositions of common carp. The results obtained from this experiment are presented in chapter 4 entitled: “Digestibility of different plant-derived oils and influence of their combination on fatty acid composition of liver and muscle in juvenile common carp (*Cyprinus carpio*)”.

Experiment 2: Growth performance and immune status in common carp Cyprinus carpio as affected by plant oil-based diets complemented with β-glucan

In order to verify hypothesis 2, this second experiment was conducted to determine the influences of LC-PUFA contents accumulated in common carp fed different lipid sources combined with β-glucan on the fish immune responses by an *in vivo* experiment and a bacterial challenge.

Concerning to the diet formulations, six experimental diets were formed using cod liver oil (CLO), linseed oil (LO), sunflower oil (SFO) without or with β-glucan (CLO+, LO+, SFO+). The candidate oils were chosen basing on their FA compositions as presented in first

experiment where CLO is always the fish oil control diet, LO is rich in ALA, and SFO was used because of its high level in n-6 PUFA, high digestibility, and its positive effect on fish FBW in the first experiment.

This experiment consisted of two continuous steps. Firstly, a nutritional trial was performed for 9 weeks where fish were fed the experimental diets to satiation. At the end of this nutritional trial, fish were intraperitoneally injected with a virulent bacterial strain of *A.hydrophila* (at dose of 5×10^8 CFU) and monitored for 10 days.

Fish specific growth rate (SGR), feed conversion rate (FCR), and survival were determined at the end of the experiment based on the data of the nutritional trial including IBW, FBW, feed intake, initial and final total numbers of fish. The humoral immune variables were measured in the fish blood plasma at the end of the feeding trial and after 48h of bacterial injection. Fish muscle and liver were collected after the nutritional trial for fatty acid composition analyses while the head kidney and liver were used for gene expression analyses. The results obtained from this experiment are shown in chapter 5 entitled: "Growth performance and immune status in common carp (*Cyprinus carpio*) as affected by plant oil-based diets complemented with β-glucan".

Experiment 3: A combined in vivo and in vitro approach to evaluate the influence of linseed oil or sesame oil and their combination on innate immune competence and eicosanoid metabolism processes in common carp (Cyprinus carpio)

In order to clarify hypothesis 3, the following experiment was performed to assess the molecular and physiological mechanisms involved in the interaction between lipid nutrition and immune responses in leukocytes isolated from common carp fed different oil sources and submitted *in vitro* to LPS stimulation.

Four experimental diets were formulated using cod liver oil (CLO), linseed oil (LO), sesame oil (SO), and a blend of linseed oil and sesame oil (SLO). The oil sources were chosen basing on the results obtained in the previous experiment where CLO is always the control diet as mentioned above; LO was used because of its abundance in ALA (n-3 PUFA precursor); SO was chosen as a plant oil-enriched in LA (n-6 PUFA precursor). In this experiment, we used SO as n-6 PUFA-enriched plant oil instead of SFO because of its positive effects in combination with LO on fish tissue LC-PUFA contents in experiment 1 and its availability in the local country (Vietnam).

This experiment was divided into two steps. Firstly, fish were fed with experimental diets to satiation for 6 weeks (Nutritional trial). At the end of the feeding period, peripheral blood mononuclear cells (PBMC) and head kidney leucocytes (HKL) were isolated from experimental fish and then exposed to LPS for 24h.

Growth performance including SGR, FCR, and survival were determined at the end of experiment. The humoral immune variables were measured in fish blood plasma as well as in cell cultured medium. Expression of genes involved in fatty acid syntheses, immune responses and eicosanoid metabolism processes were analyzed in fish liver, kidney in the nutritional trial as well as in cultured HKLs. The results of this experiment are presented in chapter 6 entitled:

"A combined *in vivo* and *in vitro* approach to evaluate the influence of linseed oil or sesame oil and their combination on innate immune competence and eicosanoid metabolism processes in common carp (*Cyprinus carpio*)".

Experiment 4: Pro- and anti-inflammatory responses of common carp (Cyprinus carpio) head kidney leukocytes to E.coli LPS as modified by different dietary plant oils

In order to verify hypothesis 4, the current experiment was carried out to evaluate the influences of free LC-PUFAs supplemented to pure plant oil-based diets imbalanced in PUFA precursors on the pro- and anti-inflammatory processes in common carp HKLs exposed to LPS.

Experimental diets were formulated using the same lipid sources as in experiment 3: CLO (cod liver oil, control diet); LO (linseed oil); SO (sesame oil); SLO, a blend of linseed oil and sesame oil (v/v, 1/1); moreover, two plant oil-based diets were supplemented with ARA (LOA, linseed oil (rich in ALA but poor in LA) + ARA,) or DHA (SOD, sesame oil (rich in LA but poor in ALA) + DHA).

This experiment was also divided into three steps including an LPS-pretest, a nutritional trial, and an *in vitro* cell culture. Firstly, the LPS-pretest was carried out to determine the cell viability and stimulating capacity of various LPS doses in common carp HKLs. Secondly, fish were fed with experimental diets to satiation for 6 weeks to modify the FA compositions in tissues (nutritional trial). At the end of the feeding period, HKLs were isolated from experimental fish and exposed to LPS for 4h and 24h.

Humoral immune variables were measured in cell cultured medium while expression of genes involved in fatty acid syntheses, innate immune responses, and pro- and anti-inflammatory responses were analyzed in cultured HKLs. The results obtained from this experiment are shown in chapter 7 entitled: "Pro- and anti-inflammatory responses of common carp *Cyprinus carpio* head kidney leukocytes to *E.coli* LPS as modified by different dietary plant oils".

Data analysis

Mean values of all variables were checked for homogeneity using univariate tests (Cochran C), when data were heterogeneous or did not have a normal distribution, a log-transformation of the data was applied and the analysis was performed on the transformed data. Data were then subjected analysis of variance (ANOVA), followed by a *LSD post-hoc* test using the diet replicate as statistical unit (according to each experiment). Differences between treatments were considered significant at P value < 0.05. All data were analyzed with the statistical package STATISTICA 5.0 (Statsorf, Inc., East 14 Street, Tulsa, USA).

Chapter 5

Growth performance and immune status in common carp (*Cyprinus carpio)* as affected by plant oil-based diets complemented with β–glucan

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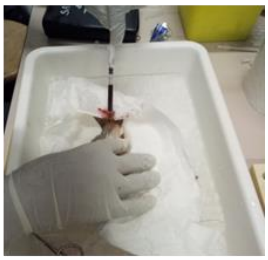

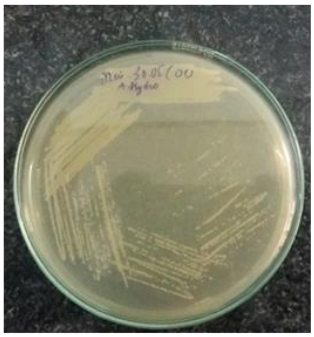

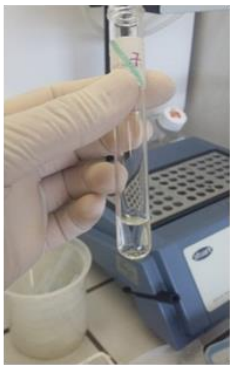


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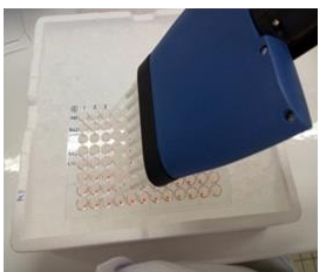

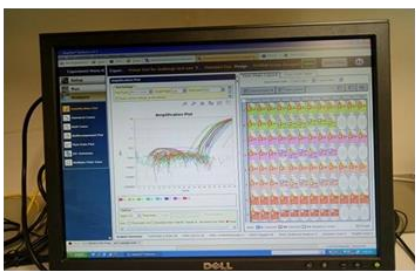
DELL

The results obtained from experiment 1 verified the first hypothesis of our study. These results are detailed in chapter 4. Briefly, the plant oil utilization did not conduct to any negative effect on fish growth, feed utilization, and survival; additionally, SFO diet even induced the highest final body weight. Lipid digestibility was influenced by dietary FA compositions with the best results in fish fed CLO and SFO-based diets. Fish fed LO-based diet have exhibited the EPA level higher than other plant oil-fed groups while the highest value of ARA levels was found in SO, SFO, and SSFO. Based on these results, we posed the scientific question of whether plant-derived oils supplemented with an immunostimulant compound could modify disease resistance, immune status, and immunocompetence of common carp and we have performed the second experiment.

This chapter presents the results obtained from experiment 2 in order to verify the second hypothesis of our study. The experimental design is detailed in the methodology chapter (Chapter 3). Shortly, common carp were fed various lipid sources supplemented without (CLO, LO, SFO) or with β-glucan (CLO+, LO+, SFO+) for 9 weeks. Fish were then injected with *A.hydrophyla* in challenge test of 10 days. The results of FA compositions in liver and muscle of fish fed different oil sources; immune status, and immunocompetence of fish fed different oils combined with β-glucan are shown in this chapter.

Abstract

Omnivorous fish species such as the common carp (*Cyprinus carpio*) are able to biosynthesize long chain polyunsaturated fatty acids (LC-PUFAs) from plant oil PUFA precursors, but the influence of the amount and quality of the LC-PUFAs biosynthesised from these oils on the immunocompetence status of the fish has received little attention. This study aims to evaluate whether the conversion of PUFA by carp induces a sufficient biosynthesis of LC-PUFA to maintain a good immunocompetence status in this species. Six iso-nitrogenous (crude protein = 39.1%) and iso-lipidic (crude lipids = 10%) diets containing three different lipid sources (cod liver oil (CLO) as fish oil; linseed oil (LO) and sunflower oil (SFO) as plant oils) were formulated with or without β–glucan supplementation at 0.25 g/kg diet. *Juvenile* carp (16.3 ± 0.6 g initial body weight) were fed a daily ration of 4% body weight for 9 weeks and then infected at day 64 with the bacteria *Aeromonas hydrophyla*. No significant differences in survival rate, final body weight, specific growth rate and feed conversion rate were observed between diets. After bacterial infection, mortality rate did not differ between fish fed CLO and plant oil-based diets, indicating that the latter oils did not affect the overall immunocompetence status of common carp. Plant oil-based diets did not alter lysozyme activity in healthy and infected fish. No negative effects of plant oils on complement activity (ACH50) were observed in healthy fish, even if both plant oil-based diets induced a decrease in stimulated fish two days after infection. Furthermore, the levels of various immune genes (*nk, lys, il-8, pla, pge, alox*) were not affected by plant oil-based diets. The expression of *pla* and *pge* genes were higher in SFO-fed fish than in CLO ones, indicating that this plant oil rich in linoleic acid (LA) better stimulated the eicosanoid metabolism process than fish oil. In response to β-glucan supplementation, some innate immune functions seemed differentially affected by plant oil-based diets. LO and SFO induced substantial LC-PUFA production, even if fish fed CLO displayed the highest EPA and DHA levels in tissues. SFO rich in LA induced the highest ARA levels in fish muscle while LO rich in α-linolenic acid (ALA) sustained higher EPA production than SFO. A significantly higher *fads-6a* expression level was observed in SFO fish than in LO ones, but this was not observed for *elovl5* expression. In conclusion, the results show that common carp fed plant oil-based diets are able to produce substantial amounts of LC-PUFA for sustaining growth rate, immune status and disease resistance similar to fish fed a fish oil-based diet. The differences in the production capacity of LC-PUFAs by the two plant oil-based diets were associated to a differential activation of some immune pathways**,** explaining how the use of these oils did not affect the overall immunocompetence of fish challenged with bacterial infection. Moreover, plant oil-based diets did not induce substantial negative effects on the immunomodulatory action of β–glucans, confirming that these oils are suitable for sustaining a good immunocompetence status in common carp.

Keywords: plant oil, immunomodulation, immunostimulant, eicosanoid metabolism process

1. Introduction

The limited availability of fish meal and fish oil is considered to be one of the major constraints in the future development of aquaculture (Burik et al., 2015; Schalekamp et al., 2016). In this context, strategies for marine fish product replacement with plant products are highly recommended. The plant products or by-products are potential material sources for fish feed production thanks to their low price and high abundance (NRC, 1993). Most plant-derived oils contain polyunsaturated fatty acids (PUFA) but no long chain PUFAs (LC-PUFA, > 18C) (Orsavova et al., 2015). Some plant-derived oils, such as linseed oil, sunflower oil or sesame oil, provide the PUFA precursors (LA and ALA) for the important n-3 (eicosapentanoic acid (EPA); docosahexaenoic acid (DHA)) and n-6 LC-PUFA (arachidonic acid (ARA)) biosynthesis (Asghar and Majeed, 2013; Bayrak et al., 2010; Popa et al., 2012; Zheljazkov et al., 2009). Most studies demonstrated that partial or total replacement of fish oil by terrestrial plant-derived oils did not influence the growth performance of freshwater fish with omnivorous or herbivorous feeding habits (Carmona-Osalde et al., 2015; Peng et al., 2016; Thanuthong et al., 2011; Turchini et al., 2011). Nonetheless, for some other species, marine and/or carnivorous, although the partial substitution of fish oil by plant oil did not induce a negative effect on fish growth (Bell et al., 2002; Montero et al., 2010; Mourente and Bell, 2006; Torrecillas et al., 2017; Zuo et al., 2015a), the total fish oil replacement in the diet was associated with a significant reduction of growth such as in Eurasian perch (Geay et al., 2015b), rainbow trout (Guroy et al., 2011; Kutluyer et al., 2017; Le Boucher et al., 2011; Mellery et al., 2017), turbot (Regost et al., 2003), sea bream (Benedito-Palos et al., 2008; Montero et al., 2010) and European sea bass (Geay et al., 2011; Torrecillas et al., 2017). Moreover, when all fish-based ingredients (including fish meal and fish oil) were replaced by plant ones, poor growth performance was reported in most freshwater species, such as rainbow trout (Le Boucher et al., 2011) and common carp (Ren et al., 2012).

The LC-PUFAs, such as ARA, EPA and DHA, play an important role in fish health and in human health (Arts et al., 2009; Oliva-Teles, 2012; Tocher et al., 2003). The sufficient supplementation of these LC-PUFAs in the diet enhances the immune response of fish (Mesa-Rodriguez et al., 2018), while a deficiency of these LC-PUFAs in plant-derived oil diets might induce fish health problems such as digestive tract deformity (Ribeiro et al., 2014), problems of gut morphology (Torrecillas et al., 2017), low bacterial resistance (Ferreira et al., 2015; Montero et al., 2010) or a reduction of some immune parameters (Conde-Sieira et al., 2018; Montero et al., 2003).

The effects of LC-PUFA insufficiency on the immune response might be linked to the deficiency in EPA and DHA, or especially ARA for eicosanoid metabolism (Calder, 2010; Tuncer and Banerjee, 2015). Eicosanoids are signalling compounds produced by cells that play a wide range of physiological functions, including in inflammatory responses (Sargent et al., 2002; Wall et al., 2010). Eicosanoids including prostaglandins and leukotrienes are produced from ARA, EPA and dihomoglinolenic acid (20:3n-6) when these FAs are released from tissue phospholipids (PL) by phospholipase A2 (PLA2) (Zhou and Nilsson, 2001). ARA is the major precursor of highly active eicosanoids while EPA produces much less active eicosanoids (Bell and Sargent, 2003; Wall et al., 2010). A study on humans showed that moderate levels of dietary essential FAs can decrease some markers of endothelial activation, and that this mechanism of action may contribute to the reported health benefits of n-3 FAs (Thies et al.,

2001). However, when the proportions of LC-PUFA-based eicosanoid actions are higher with n-6 than n-3 mediators, they cause healthy physiology to shift toward pathophysiology (Lands, 2017). Studies on mammals demonstrated that low ARA-derived prostaglandins E2 (PGE2) are associated with the stimulation of immune function, whereas high concentrations are immunosuppressive (Bell and Sargent, 2003). In fish, previous studies have focused principally on the effects of dietary FAs on the modification of the FA profile of tissues (Ma et al., 2018; Mellery et al., 2017; Teoh and Ng, 2016) or fish health (Conde-Sieira et al., 2018; Mesa-Rodriguez et al., 2018; Ribeiro et al., 2014; Torrecillas et al., 2017). It was also demonstrated that an increase in eicosanoid levels, such as thromboxane B2 and prostaglandin E2, was observed in salmon fed a diet rich in LA (known to be the precursor of ARA) (Bell et al., 1993), and an ARA-enriched diet induced changes in complex lipids and immune-related eicosanoids in zebrafish *Danio rerio* (Adam et al., 2017). However, there are few studies focusing on the extent to which omnivorous fish species can get sufficient use of precursors of PUFA from some plant oils to sustain a sufficient growth rate and immune status.

The innate immune system of fish, including cellular and humoral systems, can be stimulated by compounds such as β-glucan (Ai et al., 2007; Rodríguez et al., 2009), lipopolysaccharides (LPS) (Bich Hang et al., 2013; Selvaraj et al., 2009), bovine lactoferrin (Ibrahem et al., 2010; Khuyen et al., 2017; Mo et al., 2015), inulin (Mousavi et al., 2016) and chitosan (Anderson and Siwicki, 1994). These substances could enhance immune parameters such as lysozyme, complement, macrophage and peroxidase activities, or upregulate the expression of genes involved in the fish immune system. Among these immunostimulant, β-glucan, a polysaccharide derived from fungi or bacteria, is known to be an immunomodulatory factor (Stier et al., 2014) enhancing several inflammatory responses (Du et al., 2015; Vetvicka et al., 2013) or playing an anti-inflammatory role in some cases (Falco et al., 2012; Ruthes et al., 2013; W. J. Wang et al., 2015). The immunomodulatory actions of immunostimulant compounds may be influenced by the fluidity of cellular membranes, which is itself influenced by the FA composition in the phospholipid layer (Maulucci et al., 2016; Mironov et al., 2012; Serrazanetti et al., 2015). However, information on the influence of the amount and profile of dietary FAs on the immunomodulatory effects of immunostimulants is rather limited in fish.

The common carp is an important aquaculture species; it is the most cultured fish for human food consumption. In research, this species is an important fish for a wide range of studies focusing on physiology, such as nutrition and farming conditions (Billard, 1999), fish diseases and immunology and fish flesh quality (Böhm et al., 2014; Schultz et al., 2015; Zajic et al., 2016). Common carp is a freshwater fish that is able to biosynthesize the LC-PUFAs from PUFA precursors by a series of elongation and desaturation reactions (Oliva-Teles, 2012). Previous studies have shown that the utilization of plant oil sources rich in PUFAs, such as linseed oil, corn oil, rapeseed oil or a blend of plant oils, induced good contents of LC-PUFAs associated with higher expression levels in common carp organs of genes involved in FA metabolism, compared to those of fish fed a fish oil-based diet (Ljubojević et al., 2015; Mráz et al., 2010; Mraz and Pickova, 2011; Ren et al., 2015, 2012; Schultz et al., 2015; Trbović et al., 2013; Zajic et al., 2016). However, to our knowledge, the effects of dietary FA profiles on the immune status, and especially on the eicosanoid metabolism process, have not been investigated in this species so far. Some studies have also demonstrated that immune parameters such as lysozyme, complement, macrophage activity or the expression of genes

involved in the immune system of common carp could be stimulated by an immunostimulant supplementation, such as β-glucan, lipopolysaccharide (LPS), nucleotides from yeast RNA, chitosan or plant extracts through injection, oral administration or immersion (Herczeg et al., 2017; Kadowaki et al., 2013; Kono et al., 2004; Lin et al., 2012; Nguyen et al., 2016; Pionnier et al., 2013; Przybylska-diaz et al., 2013; Sakai et al., 2001; Watanuki et al., 2006). However, it is not known if the amount and composition of LC-PUFAs produced by omnivorous fish from dietary PUFA precursors are suitable to sustain a good immmunocompetence and modulate the response to immunostimulants, as stated above.

In this context, the present study was conducted in order to answer two questions: (1) Are common carp able to biosynthesize enough LC-PUFAs (ARA, EPA and DHA) from PUFA precursors (LA and ALA) of some plant oils to sustain a good physiological and immune status, and (2) to what extent the total replacement of fish oil by plant oils can affect the response to supplementation with an immunostimulatory compound. Based on these questions, this study aims to evaluate the influence of different lipid sources in association with β-glucans on immune parameters, tissue FA composition and expression of genes involved in FA biosynthesis, the immune system and eicosanoid metabolism processes of the common carp.

2. Materials and methods

2.1. Experimental diets

Six iso-nitrogenous (crude protein = 39.1%) and iso-lipidic (crude lipids = 10%) diets containing three different lipid sources (cod liver oil (CLO) as fish oil; linseed oil (LO) and sunflower oil (SFO) as plant-derived oils) were formulated with (CLO^+, LO^+, SFO^+) or without (CLO, LO, SFO) MacroGard β–glucan supplementation (0.25 g/kg diet). Each diet contained soluble fish protein concentrate (SFPC), wheat gluten and gelatin as protein sources. The formulation and approximate composition of the experimental diets are shown in Table 1. The studied FA composition of each diet is presented in Table 2. Ingredients were mixed and moistened with fresh water (20%) for pelleting. The 3 mm pellets were then thoroughly air-dried and stored at 4^oC.

2.2. Nutritional trial

Common carp juveniles (Initial body weight, IBW = 16.3 ± 0.7 g/fish) were obtained from the Research Institute of Aquaculture N°1 (RIA1), Vietnam. Fish were acclimated for two weeks in an indoor tank system in the wet-lab of the Faculty of Fisheries at the Vietnam National University of Agriculture. During that period, they were fed a commercial pellet for carp juveniles (Cargill, code 7434) containing 35% crude protein. After acclimation, fish were randomly distributed into 18 tanks of 120 L (3 aquariums per diet) at a density of 20 fish per tank. Fish were then fed twice a day (08.00 and 14.00) with the experimental diets at a ration of 4% body weight per day for 9 weeks. Daily feed intake was weighed and recorded to calculate feed conversion rate (FCR).

During the experimental period, the rearing conditions in the experimental system were maintained constant: temperature of 26-28^oC, dissolved oxygen at 5 mg/L, pH of 7.5 and 12h light : 12h dark photoperiod . Nitrite, nitrate and NH_3/NH_4^+ values were measured once a week and averaged 0.005, 5 and 0.05 mg/L, respectively. The tanks were siphoned daily to remove fish faeces and about 30% of the water was renewed.

Table 1. Ingredients and approximate composition of the six experimental diets

Ingredients (g/kg dry matter, DM)	Experimental diets					
	CLO	LO	SFO	CLO^{+}	LO^{+}	SFO^{+}
Soluble fish protein concentrate (SFPC)[a]	120.0	120.0	120.0	120.0	120.0	120.0
Wheat gluten[b]	300.0	300.0	300.0	300.0	300.0	300.0
Gelatin[c]	60.0	60.0	60.0	60.0	60.0	60.0
Modified starch[d]	345.0	345.0	345.0	344.75	344.75	344.75
Cod liver oil[e]	100.0	0	0	100.0	0	0
Sunflower oil[f]	0	0	100.0	0	0	100.0
Linseed oil[g]	0	100.0	0	0	100.0	0
Vitamin premix[h]	10.0	10.0	10.0	10.0	10.0	10.0
Mineral premix[i]	65.0	65.0	65.0	65.0	65.0	65.0
MacroGard (β-glucans)[j]	0	0	0	0.25	0.25	0.25
Total	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0
Crude protein, CP (% DM)	39.1	39.1	39.1	39.1	39.1	39.1
Crude fat, CF (% DM)	10.0	10.0	10.0	10.0	10.0	10.0
Gross energy, GE (MJ/Kg DM)	19.1	19.1	19.1	19.1	19.1	19.1
CP/GE (g/MJ)	20.5	20.5	20.5	20.5	20.5	20.5
CF/GE (g/MJ)	5.2	5.2	5.2	5.2	5.2	5.2

Experimental diet nomenclature: CLO: cod liver oil-based diet, LO: linseed oil-based diet and SFO: sunflower oil-based diet with (CLO^{+}, LO^{+}, SFO^{+}) or without (CLO, LO, SFO) β–glucan supplementation

[a]Cook Carp Concept, 56 Rue de Metz, 57130 Jouy-aux-Arches, France

[b,c,g] Sigma-Aldrich, St Louis, MO, USA

[d]Baaboo Food, Ho Chi Minh City, Vietnam

[e]Mosselman s.a., Route de Wallonie, B-7011 Ghlin, Belgium

[f]Simply Oil, Cai Lan Oils & Fats Industries Co., Ltd, Vietnam[i]

The vitamin premix was formulated following (Abboudi et al., 2009) *(to provide g/kg mixture, except as noted): retinyl acetate (1 500 000 IU/g), 0.67; ascorbic acid, 120; cholecalciferol (4 000 000 IU/g), 0.1; tocopheryl acetate (1 000 IU/g), 34.2; menadione, 2.2; thiamin, 5.6; riboflavin, 12; pyridoxine, 4.5; calcium-panthotenate, 14.1; p-aminobenzoic acid, 40; vitamin B12, 0.03; niacin, 30; biotin, 0.1; choline chloride, 350; folic acid, 1.5; inositol, 50; canthaxanthin, 5; astaxanthin, 5; butylated hydroxytoluene, 1.5; butylated hydroxyanisole, 1.5; α-cellulose, 325.*

[j]The mineral premix was formulated following (Abboudi et al., 2009) *(to provide g/kg mixture, except as noted): $CaHPO_4{\cdot}2H_2O$, 295.5; $Ca(H_2PO_4)2{\cdot}H_2O$, 217; $NaHCO_3$, 94.5; $Na_2SeO_3{\cdot}5H_2O$, 11 mg; KCl, 100; NaCl, 172.4; KI, 0.2; $MgCl_2$, 63.7; $MgSO_4$, 34.3; $MnSO_4{\cdot}4H_2O$, 2; $FeSO_4{\cdot}4H_2O$, 10; $CuSO_4{\cdot}5H_2O$, 0.4; $ZnSO_4{\cdot}7H_2O$, 10.*

2.3. Challenge test

A strain of *Aeromonas hydrophila* was originally isolated and identified from infected common carp and identified by the Centre of Research and Development in Biotechnology, Hanoi University of Science and Technology, Vietnam, according to the protocol of (Rashid et al., 2014). The bacterial culture process was described in (Nguyen et al., 2016). The median lethal dose LD50 was determined by intraperitoneal injection with doses of 10^6, 10^7, 10^8 and 10^9 CFU/fish and the results showed that the LD50 was 5.01×10^8 CFU/fish, for fish of 30g. One day after the end of the nutritional trial, at day 64, fish were divided into two batches; one group was intra-peripherally injected with *A. hydrophila* with a dose of 5.01×10^8 CFU/fish and the other group with the bacterial medium culture Tryptic Soy broth (TSB; Merck, Darmstadt, Germany) only. Non-supplemented and β-glucan supplemented fish were then monitored over a period of 10 days and the daily mortality was recorded. The bacterial contamination was confirmed by the re-implantation of the infected fish kidney samples on the nutrient agar medium and bacterial colony descriptions were followed (Agger et al., 1985).

2.4. Sample collection

After 9 weeks of rearing (D63), the total fish number and body weight were recorded to determine the survival rate (SR) and specific growth rate (SGR), respectively. At the end of the growth trial and after two days (D65) of bacterial challenge test, three fish per aquarium were randomly selected and anesthetised with clove oil (50 µL/L, Sigma-Aldrich). Heparin blood plasma was individually sampled for lysozyme and complement (ACH50) activities, fish liver and dorsal muscle were dissected to analyse the FA composition, while fish kidney and liver were collected for gene expression analyses. The tissue samples were snap frozen in liquid nitrogen and then stored at -80°C.

2.5. Sample analysis

2.5.1. Fatty acid analyses

The experimental diets were homogenised and the lipids were extracted with chloroform/methanol (2:1, *v:v*) according to the Folch method (Folch et al., 1957), edited by Christie (1982) while lipids of fish liver and dorsal muscle (3 fish per tank) were extracted by chloroform/methanol/water (2:2:1.8, *v:v:v*) following a method adapted from Bligh and Dyer (1959).

Table 2. Fatty acid composition (% of total identified fatty acids) in the experimental diets

	Diet					
	CLO	LO	SFO	CLO$^+$	LO$^+$	SFO$^+$
C6:0	0.2	0.2	0.0	0.3	0.5	0.4
C8:0	0.1	0.0	0.0	0.0	0.0	0.0
C10:0	0.1	0.1	0.1	0.1	0.1	0.1
C12:0	0.1	0.1	0.1	0.1	0.1	0.1
C14:0	3.7	0.5	0.5	4.1	0.6	0.9
C15:0	0.3	0.1	0.1	0.3	0.1	0.1
C16:0	12.7	7.9	8.7	12.4	7.9	8.7
C17:0	0.3	0.1	0.1	0.3	0.1	0.1
C18:0	2.7	3.3	3.3	2.5	3.3	3.2
C18:1n-9 (OA)	20.8	21.5	25.4	19.6	21.8	25.3
C18:2n-6 (LA)	11.5	22.0	53.3	9.9	22.8	48.0
C18:3n-3 (ALA)	4.4	39.3	1.5	2.1	37.3	1.6
C20:4n-6 (ARA)	0.5	--	--	0.5	--	--
C20:5n-3 (EPA)	6.5	--	--	7.6	--	--
C22:6n-3 (DHA)	9.0	--	--	10.5	--	--
SFA	20.4	12.8	14.0	20.4	13.1	14.7
MUFA	43.0	23.5	28.6	43.8	23.9	30.9
C18-PUFA	36.6	63.7	57.4	35.8	62.9	54.5
C18-PUFA n-6	32.5	43.5	78.8	29.6	44.6	73.3
C18-PUFA n-3	17.5	61.4	55.0	13.9	60.3	49.9
LC-PUFA	18.8	2.4	2.4	21.7	2.7	4.5
LC-PUFA n-6	1.2	0.3	0.3	1.3	0.3	0.3
LC-PUFA n-3	17.6	2.1	2.1	20.4	2.4	4.2
n3/n6 ratio	1.8	1.9	0.1	2.2	1.7	0.1
ALA/LA	0.4	1.8	0.03	0.2	1.6	0.03

Experimental diet nomenclature: See table 1. OA: oleic acid; LA: linoleic acid; ALA: α-linolenic acid; ARA: arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosapentaenoic acid; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: C18-polyunsaturated fatty acids; LC-PUFA: long chain polyunsaturated fatty acids.

Briefly, the extracted lipids were converted into FA methyl esters via methylation and subsequently separated by gas chromatography (GC) and quantified following Mellery et al. (Mellery et al., 2017). The GC trace (Thermo Scientific, Milan, Italy) was equipped with a capillary column of 100 m × 0.25 mm, 0.2 µm film thickness (RT 2560, Restek, Bellefonte, PA, USA). The gas vector (hydrogen) was injected at a pressure of 200 kPa. The flame ionisation detector (FID, Thermo Scientific) was kept at a constant temperature of 255°C. The oven temperature program was as detailed in (Mellery et al., 2017). Each peak was identified by comparison of retention times with those for pure methyl ester standards (Larodan, Solna, Sweden) and Nu-Check Prep (Elysian, Minnesota, USA). Data were processed using ChromQuest software 3.0 (Thermo Finnigan, Milan, Italy). The final results are expressed in percentageof total identified fatty acids.

2.5.2. Immune parameter analyses

Lysozyme activity was determined according to the protocol of Ellis (Ellis, 1990) adapted for common carp. Heparin blood plasma (30 µL) was individually added in triplicate to 30 µL of PBS buffer (phosphate-buffered saline, pH 6.2). The 100 µL-bacterial suspension of *Micrococcus lysodeikticus* (Sigma-Aldrich) (200 mg/L in 0.05 M NaH_2PO_4, pH 6.2) was then added to the mix of plasma and PBS buffer. Two readings at 530 nm wave length were taken with a spectrophotometer after 30 s and 4.5 min of shaking. The lysozyme activity unit (U/mL) was defined as the amount of enzyme causing a decrease in absorbance of 0.001/min.

The protocol to determine the complement activity was described in (Saha et al., 1993) and adapted for common carp. Accordingly, blood plasma was added by a series of dilutions with veronal buffer (VCM-F, BioMérieux, Marcy l'Étoile, France) to a 96-well round bottom plate. Wells were then filled with 10 µL of 3% rabbit blood cells (RaRBC, BioMérieux) (70 µL total volume for each well). Samples were incubated at 27°C for 2h and centrifuged (3000×g, 5 min, 4°C) to collect the supernatant. Then, 35 µL of supernatant was measured the absorbance at 405 nm. The haemolysin (HLY) was recorded as the highest dilution of plasma showing complete lysis. The ACH50 value was defined as the reciprocal of the plasma dilution which induced 50% haemolysis of RaRBC.

2.5.3. Gene expression analyses

The total RNA of liver and kidney were individually extracted from a batch of three fish for each tank using 1 mL trizol (Extract-all®, Eurobio, Courtaboeuf, France). The quality of extracted RNA was checked using a Nanodrop 2000 spectrophotometer (Thermo-Fischer Scientific, Waltham, MA, USA) and electrophoresis on a 1.2% agarose gel. Each individual RNA sample was then treated using a RTS DNAse™ kit (MO BIO Laboratories, Carlsbad, CA, USA) to avoid DNA contamination. Then, 1 µg of total RNA was reverse transcribed to cDNA in using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). The cDNA was then diluted with ultrapure water (Invitrogen™ UltraPure™ DNase/RNase-Free Distilled Water, Thermo-Fisher scientific) and used for real-time qPCR to determine gene expression levels. Expression of *nk* (natural killer cell enhancing factor), *lys* (lysozyme), *il8* (interleukin 8), *elovl5* (elongase very long delta 5), *fads6-a* (fatty acid desaturase delta 6), *pla* (phospholipase A2), *pge* (prostaglandin E2 synthase) and *alox* (Arachidonate 5-lipoxygenase) genes were determined using specific primers that were designed on Primer3 software and re-checked for quality on Amplifix software against sequences of the common

carp published on Genbank (Table 3). The efficiency of each gene was confirmed before analysis. The *40s* (40S ribosomal protein) and *18s* (18S ribosomal RNA) (Zhang et al., 2016) genes were used as housekeeping genes. The amplification of cDNA was conducted in triplicate using an iQ™ SYBR® Green Supermix kit (Bio-Rad Laboratories, Hercules, CA, USA). Thermal cycles and fluorescence detection were carried out using a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) under the following conditions: 10 min of initial denaturation at 95°C, followed by 40 cycles of 95°C for 30 s and 60°C for 30 s. For analysis, a standard curve produced from a pool of cDNA from all samples was included to calculate the PCR efficiency and to normalise the transcript levels. The relative levels of RNA were quantified for each gene by densitometry, which was performed by measuring the photostimulated luminescence values using StepOne Software v2.1. Ratios of candidate gene/housekeeping gene products were subsequently calculated for each gene and used to assess the differences in expression levels between experimental groups

Table 3. Primer sequences for amplification of candidate genes involved in the immune system, FA biosynthesis and eicosanoid metabolism processes in common carp.

Genes	Function	Genbank No.	Primer sequence
Immune genes			
il8	Cytokines	EU011243	Fw: CGCTGCATTGAAACTGAGAG Rv: TTAACCCAGGGTGCAGTAGG
nk	Natural killer cell enhancing factor	AB048789	Fw: TGTGATGCCAGATGGACAGT Rv: CCTTGTTTCCGAGGTGTGTT
lys	Lysozyme	AB027305	Fw: GTGTCTGATGTGGCTGTGCT Rv: GAACGCACTCTGTGGGTCTT
Fatty acid biosynthesis genes			
fads6-a	Desaturase delta 6	(Ren et al., 2012)	Fw: ATCGGACACCTGAAGGGAGCG Rv: CATGTTGAGCATGTTGACATCCG
elovl5	Elongase delta 5	KF924199	Fw: AGGAGAGGCTGACAACAGGA Rv: CAGGAAGGTGATCTGGTGGT
Eicosanoid metabolism genes			
pla	Secreted phospholipase	KF793834	Fw: CTGCATGACAAGTGATGAGCAA Rv: CTGGTGCTCAAATCCATCAGGT
pge	Prostaglandin E synthase 2	XM_019098948	Fw: CAAGGAATTCATGGGAGGCGATCA Rv: CACACGTCGGTACCAGTTCTTCA
alox	Arachidonate 5-lipoxygenase	XM_019066935	
Housekeeping genes			
40s	40S ribosomal protein	AB012087 (Zhang et al., 2016)	Fw: CCGTGGGTGACATCGTTACA Rv: TCAGGACATTGAACCTCACTGTCT
18s	18S ribosomal RNA	FJ710826 (Zhang et al., 2016)	Fw: GAGTATGGTTGCAAAGCTGAAAC Rv: AATCTGTCAATCCTTTCCGTGTCC

2.6. Data presentation and statistical analysis

The husbandry parameters were calculated as follows:
Survival rate (SR, %) = 100 × final number of fish/initial number of fish
Specific growth rate (SGR, %/day) = 100 × (Ln (FBW) – Ln (IBW))/ΔT

where FBW and IBW are final and initial body weights, respectively, and ΔT is the number of days of the growth trial
FCR = (final biomass – initial biomass + dead biomass)/feed intake

Mean values of all variables were checked for homogeneity by univariate tests, and then subjected to a two-way analysis of variance (ANOVA 2) followed by a *LSD post-hoc* test using the tank replicate as statistical unit (n = 3). Differences between treatments were considered significant at *P* value < 0.05. All data were analysed with the statistical package STATISTICA 5.0 (Statsorf, Inc., East 14 Street, Tulsa, USA).

3. Results

3.1. Growth and feed utilization

After nine weeks of feeding, the husbandry parameters, namely SGR, SR and FCR, were calculated and results are presented in Table 4.

Table 4. Husbandry parameters of experimental fish fed different plant oil diets with or without β-glucans after 9 weeks of rearing.

Diet / *Parameters*	CLO	LO	SFO	CLO^+	LO^+	SFO^+
IBW (g/fish)	15.6 ± 0.4	16.9 ± 0.5	16.4 ± 0.5	16.0 ± 0.7	16.4 ± 0.8	16.2 ± 0.6
FBW (g/fish)	34.1 ± 2.1	32.7 ± 1.3	33.6 ± 2.2	36.2 ± 7.3	34.7 ± 2.2	31.7 ± 4.4
SGR (%/day)	1.2 ± 0.2	1.0 ± 0.1	1.1 ± 0.2	1.3 ± 0.3	1.2 ± 0.1	1.3 ± 0.2
FCR	1.84 ± 0.4	2.01 ± 0.3	2.23 ± 0.5	2.03 ± 0.5	1.97 ± 0.3	1.81 ± 0.3
SR (%)	98.7 ± 2.3	94.7 ± 2.3	97.3 ± 2.3	97.3 ± 2.3	96.0 ± 6.9	97.3 ± 4.6

Values were represented by means ± SD. CLO: cod liver oil-based diet, LO: linseed oil-based diet and SFO: sunflower oil-based diet with (CLO^+, LO^+, SFO^+) or without (CLO, LO, SFO) β–glucan supplementation. IBW: initial body weight; FBW: final body weight; SGR: specific growth rate; FCR: feed conversion rate; SR: survival rate. Data were transformed in Log for final body weight; Arcsine ($\sqrt{X}$) for survival rate before statistical analysis. Values with no common superscript letter within the same row denote significant differences (P <.05)

No differences between groups were observed for all parameters. The FBW was two times higher than IBW (33.8 g *vs.* 16.3 g), with a SGR ranging from 1.1 to 1.3%/day. A high SR was observed in all treatments (ranging from 94.7 to 98.7%), suggesting that the rearing conditions were suitable for common carp requirements.

3.2. Fatty acid composition in carp liver and muscle and expression of genes involved in FA biosynthesis processes

Dietary FA composition varied with the dietary oil sources (Table 2). Of note, LA was abundant in diets containing SFO (four times higher than in CLO diets and two times higher than in LO diets) while ALA was abundant in LO-based diets (11 and 24 times higher than in of CLO- and SFO-based diets). The LC-PUFAs such as ARA, EPA and DHA were only provided for fish fed CLO-based diets. The LO and SFO-based diets were rich in PUFAs whereas CLO-based diets contained a high level of LC-PUFAs. The n-3/n-6 ratio in LO diets was comparable to that of CLO diets (ranging from 1.7 to 2.2) and higher than in the SFO diets (n-3/n-6 = 0.1). The ALA/LA ratio value was the lowest in the SFO (0.03) diets compared to CLO (0.2 and 0.4) and LO (1.6 and 1.8) diets.

At the end of the experimental feeding period, we observed significant differences in the FA levels of carp liver and muscle between experimental conditions (P <.05). No influences of β-glucan supplementation were found on the FA profiles of liver and dorsal muscle from common carp.

In liver, a difference was observed in all the FA types (Figure 1a). The highest level of SFA was found in the CLO group and the same results were recorded for MUFA and LC-PUFA contents (P < .05). In contrast, the PUFA contents in LO and SFO groups reached a higher value than in the CLO group (P < .05). Regarding the essential PUFA levels, we found significant differences in LA and ALA levels (P < .05). In contrast, the major MUFA, namely OA, remained at a similar level in all treatments (Figure 1b).

The highest value of LA levels was observed in SFO fish, while that of the CLO group was the lowest, the LO-fed fish being in an intermediate position. ALA was abundant in LO fish but very low in other treatments (P < .05). The major LC-PUFA presented different levels between experimental conditions (Figure 1c). The level of ARA in the SFO group (1%) was significantly higher than those in the CLO (0.2%) and LO (0.3%) groups. EPA and DHA levels were the highest in CLO fish while the lowest value was found in the SFO group, intermediate value being observed in LO-fed fish (P < .05). The n3/n6 ratio varied around 1.2 (Figure 1c) for fish fed CLO- and LO-based diets, and was significantly higher (P < .05) than in the SFO group (around 0.1).

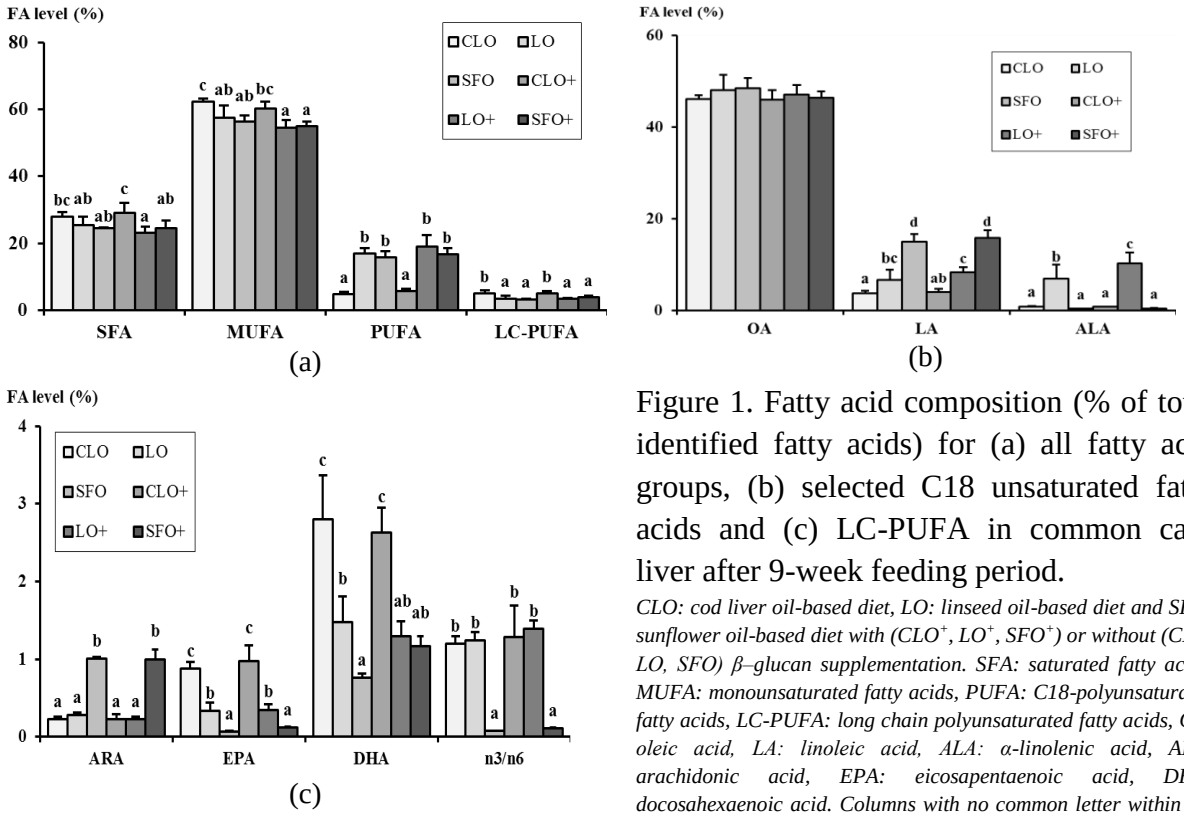

Figure 1. Fatty acid composition (% of total identified fatty acids) for (a) all fatty acid groups, (b) selected C18 unsaturated fatty acids and (c) LC-PUFA in common carp liver after 9-week feeding period.

CLO: cod liver oil-based diet, LO: linseed oil-based diet and SFO: sunflower oil-based diet with (CLO$^+$, LO$^+$, SFO$^+$) or without (CLO, LO, SFO) β–glucan supplementation. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: C18-polyunsaturated fatty acids, LC-PUFA: long chain polyunsaturated fatty acids, OA: oleic acid, LA: linoleic acid, ALA: α-linolenic acid, ARA: arachidonic acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid. Columns with no common letter within the same group of FAs denote significant differences (P < .05) (*)

In dorsal muscle, the FA composition showed similar trends than in the liver, with diet-related differences found in all the FA groups (Figure 2a), essential PUFA (Figure 2b) and the main LC-PUFA (Figure 2c). SFA and MUFA contents in dorsal muscle of fish fed the CLO-based diet were higher than for the groups fed LO and SFO diets; a similar result was observed for

LC-PUFA content. In contrast, the contents in C18-PUFA were much higher in the muscle of fish fed plant oil-based diets than CLO-fed fish ($P < .05$, Figure 2a). Regarding the levels of OA and essential PUFA (Figure 2b), we observed an increase in OA levels with the plant oil-based diets, the difference being significant in the SFO^{+} condition ($P = .03$). Differences were much more striking for LA and ALA levels in muscle. The highest values for LA were observed in the SFO groups, while they were the lowest for fish fed the CLO ($P < .05$). Intermediate levels were observed in LO-fed fish. As for ALA, the muscle of fish fed a LO diet presented much higher levels, as compared to the CLO and SFO conditions for which the ALA levels remained very low ($P < .05$).

Results concerning LC-PUFA (ARA, EPA and DHA) levels were also significantly different ($P < .05$) between experimental conditions (Figure 2c). The ARA levels in the SFO groups were significantly higher ($P < .05$) than those found in the CLO or LO conditions. In contrast, the DHA levels in the muscle of fish fed the SFO and LO diets were significantly lower than for the CLO groups. The EPA contents in the muscle of CLO-fed fish were about 2.5 times higher than in the muscle of LO-fed fish and 7 times higher than in the SFO conditions. Accordingly, the EPA levels in the muscle of fish fed the LO-diets were about 3 times higher than in the corresponding tissue of fish fed the SFO-diets. Interestingly enough, the n-3/n-6 ratios were close to 1.7 for the CLO and LO groups, while being very low (around 0.1) in the muscle of SFO-fed fish ($P < .05$, Figure 2c).

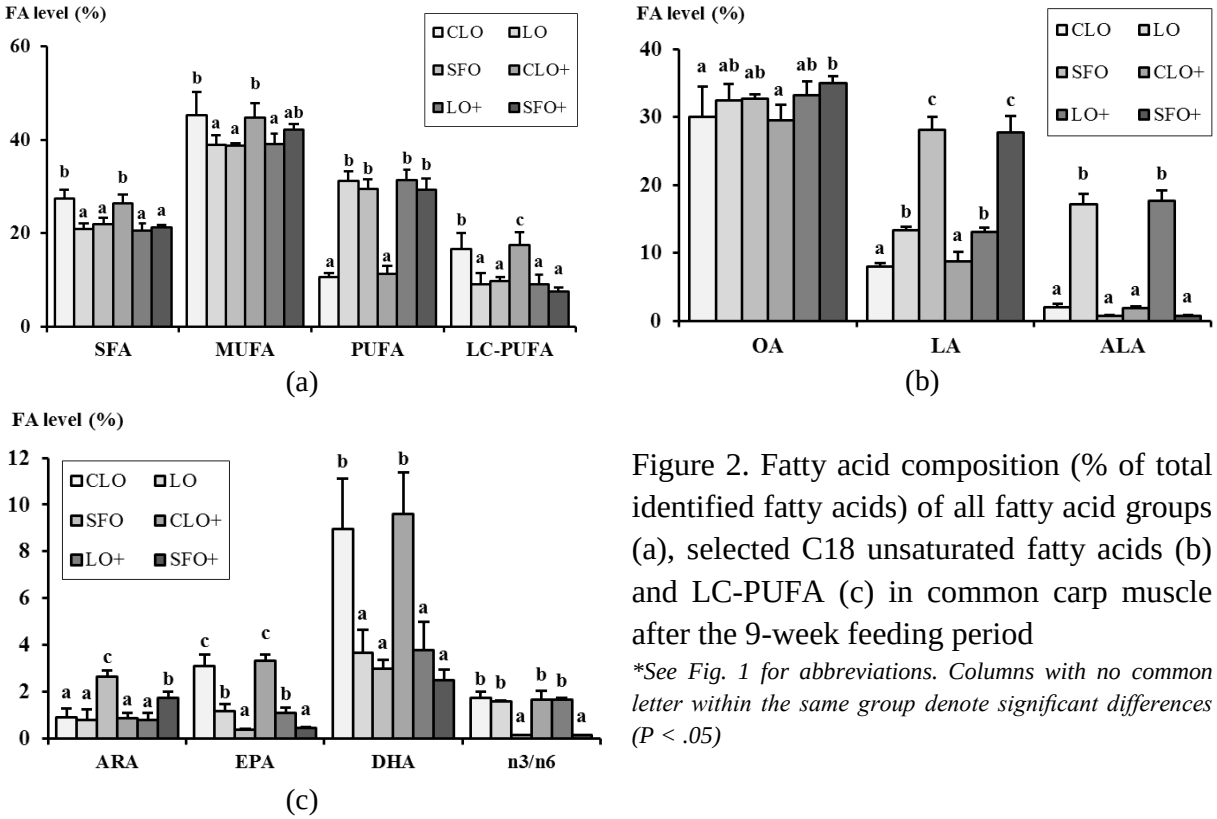

Figure 2. Fatty acid composition (% of total identified fatty acids) of all fatty acid groups (a), selected C18 unsaturated fatty acids (b) and LC-PUFA (c) in common carp muscle after the 9-week feeding period

**See Fig. 1 for abbreviations. Columns with no common letter within the same group denote significant differences (P < .05)*

The expression of genes related to FA biosynthesis processes (*fads-6a, elovl5*) was determined in fish liver tissue (Figure 3). The expression levels of *fads-6a* and *elovl5* genes were comparable between fish fed the two plant oil-based diets and those receiving CLO with or without β-glucan supplementation. *Fads-6a* was up-regulated in SFO-fed fish in comparison to LO-fed fish, but this difference was not observed when the feeding treatment

included β-glucans. Such an interaction between SFO and β-glucan supplementation was not observed for *elovl5* expression.

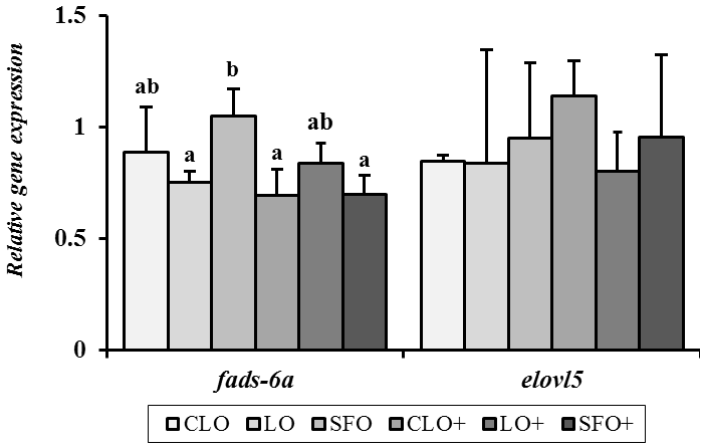

Figure 3. Expression of genes involved in fatty acid biosynthesis in common carp

**See Fig. 1 for abbreviations. Columns with no common letter within the same group denote significant differences (P < .05)*

3.3. Immune response and expression of related immune genes

After a 10-day challenge test with an *A. hydrophyla* dose of 5.01×10^8 CFU/mL, the observed mortality was lower than 50% and varied from 12.6 to 13.7% with no difference between experimental conditions either for non-supplemented fish or β-glucan treated ones.

On D63 (healthy fish), in the groups fed with diets without β-glucans, the plant oil-based diets did not negatively affect plasma lysozyme activity (Figure 4). SFO-fed fish even displayed higher values ($P < .05$) than CLO fish. In contrast, SFO- or LO-based diets with β-glucan supplementation lowered the lysozyme activity, as fish fed SFO+ or LO+ displayed lower lysozyme activities than fish fed CLO+. In infected fish (D65), plant oil-based diets did not impair the lysozyme activity, which was even higher in fish fed LO than a CLO-based diet. Moreover, the lysozyme response with β-glucan supplementation was comparable between LO+ and CLO+ groups, but was the lowest ($P < .05$) in fish fed SFO+, indicating a negative interaction with SFO.

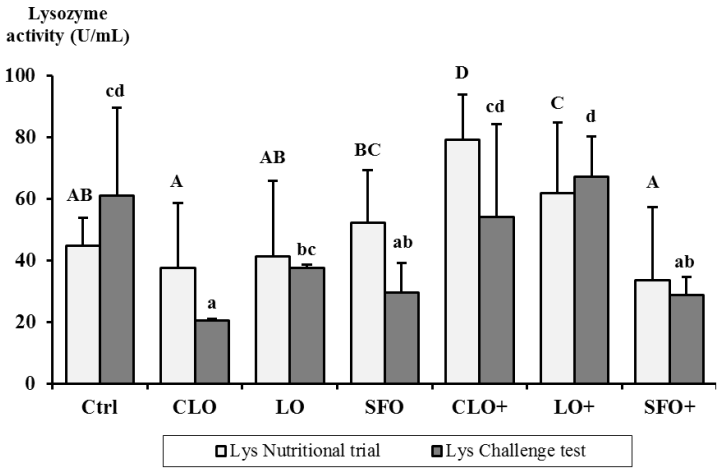

Figure 4. Lysozyme activity in the blood plasma of common carp at the end of the nutritional trial (D63) and after two days of challenge test (D65)

**See Fig. 1 for abbreviations; Ctrl: fish at D0 of feeding trial and non-injected fish with bacteria in challenge test. Values are represented by means ± SD. Values with no common letter within columns denote significant differences between diets (P < .05)*

Regarding the results of alternative complement activity (ACH50) (Figure 5), no negative effects of plant oil-based diets were observed without or with β-glucans as values were comparable between all experimental groups in healthy fish on D63. β-glucan supplementation did not induce any alteration in ACH50 response whatever the oil source. ACH50 values were lowered by bacterial infection in all experimental groups, especially when plant oils were combined with β-glucans as for fish fed LO+ and SFO+ compared to fish fed CLO+ ($P < .05$).

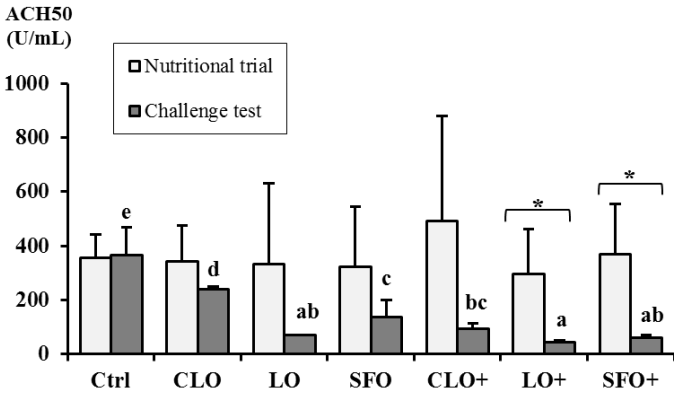

Figure 5. Alternative complement activity (ACH50) in the blood plasma of common carp at the end of the nutritional trial (D63) and after two days of challenge test (D65)

**See Fig. 1 for abbreviations; Ctrl: fish at D0 of feeding trial and non-injected fish with bacteria in challenge test. Values are represented by means ± SD. Values with no common letter within columns denote significant differences between diets (P < .05). Symbol (*) denotes a significant difference within a diet group, before and after the challenge test (P < .05)*

The expression of several immune genes (*nk, lys* and *il8*) was assayed in kidney (Figure 6). The *nk* gene expression level in SFO was higher than in CLO and LO fish, while this difference was not found in groups fed additionally with β-glucans. The dietary β-glucan supplementation enhanced the expression of *nk* in fish fed a CLO-based diet whereas any stimulation was observed for LO+ and SFO+ groups. Regarding *lys* gene expression, the level was comparable between groups without β-glucans, while the response to β-glucans was altered in LO+ fed fish but not in SFO+ ones. Concerning the expression of the *il8* gene, no negative effect of plant oils was observed with or without β-glucans and this supplementation induced *il8* up-regulation in only CLO-fed fish.

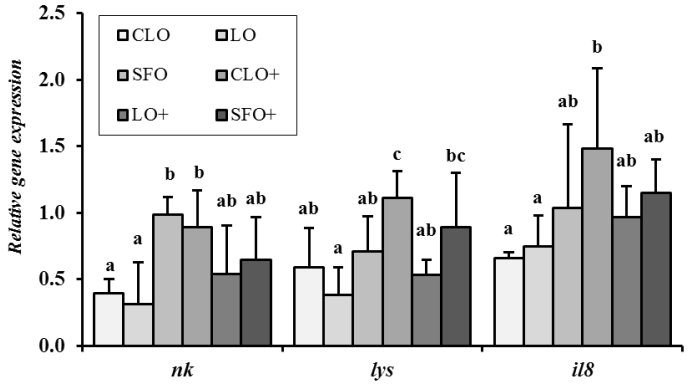

Figure 6. Expression of related immune genes in common carp kidney

**See Fig. 1 for abbreviations; nk: natural kille cell enhancing factor; lys: lysozyme; il8: interleukin 8. Columns with no common letter within the same group denote significant differences (P < .05)*

In liver tissues, both β-glucan supplementation and dietary FA profiles significantly affected the expression of *pla* and *pge* genes, while no differences were found in *alox* gene expression (Figure 7).

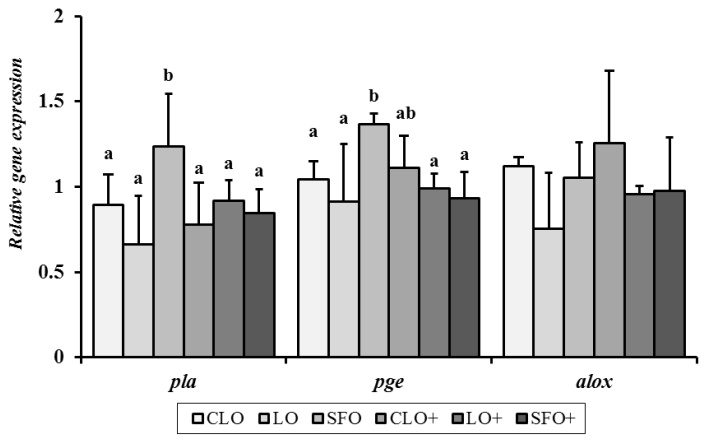

Figure 7. Expression of genes involved in eicosanoid metabolism processes in common carp liver

**See Fig. 1 for abbreviations. pla: phospholipase A2; pge: prostaglandin E synthase 2; alox: arachidonate 5-lipoxygenase. Columns with no common letter within the same group denote significant differences (P < 0.05).*

Specifically, we found an up-regulation of *pla* and *pge* gene expression in the SFO group compared with CLO- and LO-fed fish without β-glucans ($P < .05$); but no modulation of the expression of these genes was observed whatever the oil source after β-glucan supplementation as the expression levels did not differ between CLO+, LO+ and SFO+ groups.

4. Discussion

4.1. Growth parameters

No growth differences were recorded between the experimental diets. This indicates that the lower amounts of LC-PUFAs produced by common carp fed with LO or SFO diets compared to fish fed with a CLO diet did not negatively influence the fish growth performance. As reported in the introduction, a similar trend was observed in previous studies, suggesting that a total plant oil utilization can generally be used in freshwater or omnivorous fish species. However the total replacement of FO by plant-derived oil in marine or carnivorous fish diet frequently induces a reduction of growth performance (Benedito-Palos et al., 2008; Geay et al., 2015a; Guroy et al., 2011; Kutluyer et al., 2017; Le Boucher et al., 2011; Regost et al., 2003; Torrecillas et al., 2017). In our experiment, the SGR values (from 1.0 to 1.3%/day) were similar to those of the study in (Zajic et al., 2016) (about 1.3%/day) and higher than those reported in (Ren et al., 2012) (0.4%/day) for the same species and the same developmental stage (juveniles of 40 to 50 g). In the current study, we used soluble fish protein concentrate as one of the protein sources. This ingredient does not contain the fish oil usually present in commercial fish meal (containing from 5 to 10% fish oil (Jensen et al., 1990)). It is also interesting to note that the profile of LC-PUFAs did not affect the growth rate, as fish fed a CLO diet displayed the highest levels of EPA and DHA while fish fed SFO only produced higher levels of ARA.

We also observed that supplementation with β-glucans did not improve the husbandry parameters. Similar observations were found in previous studies with common carp where the authors used different compounds such as β-glucans (Selvaraj et al., 2009), chitosan (Lin et al., 2011) or May chang *Litsea cubeba* leaf powder (Nguyen et al., 2016) as dietary immunostimulants.

4.2. Fatty acid composition of liver and muscle and related gene expression

FA profiles in common carp tissues reflected those of the respective diets. Tissues from fish fed a CLO diet were rich in EPA and DHA, whereas tissues of LO-fed fish were rich in ALA, and SFO-fed fish were rich in LA. Nonetheless, tissues from fish fed plant oil-based diets contained substantial levels of LC-PUFA, with higher levels of ARA for the SFO conditions, and higher levels of EPA in the LO conditions as compared to the SFO conditions. These two observations indicate that common carp has an active capacity for biosynthesis of LC-PUFA from the precursors contained in plant oils, enabling them to have enough essential FAs to sustain optimal growth performance. Similar findings were previously reported by several authors (Ren et al., 2012; Schultz et al., 2015; Zajic et al., 2016; Zupan et al., 2016) on the same species, suggesting a specific ability of common carp to biosynthesize ARA from LA, and EPA from ALA. The levels of EPA and DHA in the liver and muscle of fish fed a CLO diet (0.9 and 2.8% in liver and 2.8 and 9% in muscle) were higher than those reported in (Fontagné et al., 1999) (0.8 and 2.4% respectively), (Mráz and Pickova, 2009) (1.16 and

5.26% respectively) and (Stancheva and Merdzhanova, 2011) (0.85 and 1.63% respectively) in the same species.

In the present study, a lower dietary ALA/LA ratio (0.03 in SFO-based diet *vs.* 0.4 in CLO and 1.8 in LO diets) is associated to a lower EPA level. Similar observations were also reported in Murray cod *Maccullochella peelii* (Senadheera et al., 2010) and juvenile tambaqui *Colossoma macropomum* (Paulino et al., 2018). Apart from a good ALA/LA ratio, the LO and CLO diets also induced the highest n-3/n-6 ratios in common carp muscle (around 1.7), which is higher than those previously reported in the same species in (Stancheva and Merdzhanova, 2011), (Mráz et al., 2012) and (Hong et al., 2014). The present results indicate that these two oils support the production of high quality fish fillet for human consumption, as far as the n3/n6 ratio is concerned. Indeed, the dietary n-3/n-6 ratios are implicated in controlling markers of metabolic parameters, including insulin sensitivity, inflammation, lipid profiles and adiposity (Burghardt et al., 2010). According to several authors (Bhardwaj et al., 2016; Gómez Candela et al., 2011; Simopoulos, 1991) humans have been evolutionary adapted to a diet with a n-3/n-6 ratio close to 1. This observation supports the suitability of linseed oil as a plant-derived oil to substitute fish oil in carp feed, not only in terms of carp culture performance, but also from a human nutrition perspective.

Regarding the results of FA profiles in diets and liver, the LA (a precursor of ARA) levels in SFO diets reached about 53%, while ALA (a precursor of EPA) in LO diets reached 39%. However, the ARA levels in SFO-fed fish liver were around 1%, whereas EPA levels in LO-fed fish liver were limited to 0.3%. The lower level of anabolic conversion in the case of ALA-EPA may be linked to the lower accumulation of ALA in the tissues, as compared to LA, probably because ALA is more prone to be used as an energetic substrate as it has been reported in mammals (Fu and Sinclair, 2000; Ide et al., 1996; Leyton et al., 1987). In addition, a link may also be made with the higher expression level of *fads-6a* in SFO fish, suggesting higher desaturase enzyme activity in the FA biosynthesis pathway of SFO fish than LO or CLO fish. Nonetheless, the expression of *elovl5* did not differ between experimental treatments, although the LC-PUFA was influenced by dietary FA composition.

4.3. Immune status and immunomodulatory response

We observed a marked influence of dietary lipid source and β-glucan supplementation on plasma lysozyme activity ($P < .05$) (Figure 5) at the end of the nutritional trial (D63) and after the challenge test (D65). Lysozyme is a bacteriolytic enzyme that is widely distributed throughout the body and is part of the nonspecific defense mechanisms in most animals (Uribe et al., 2011a). Similar results were found in some studies with common carp fed diets containing nucleotides isolated from yeast RNA (Sakai et al., 2001), chitosan (Gopalakannan and Arul, 2006; Lin et al., 2012), chitin (Gopalakannan and Arul, 2006), plant extract (Nguyen et al., 2016), lipopolysaccharide (Kadowaki et al., 2013; Selvaraj et al., 2009) or β-glucans (Lin et al., 2011; Pionnier et al., 2013; Selvaraj et al., 2005). The highest lysozyme activity was measured in CLO+ fish plasma (79 U/mL), where it was more than two times higher than the values reported in (Lin et al., 2011) (about 30 U/mL after 56 days of rearing) with dietary β-glucans at a much higher dose than in our experiment (900 mg/kg diet instead of 250 mg/kg diet in our work), or in (Lin et al., 2012) (about 40 U/mL) where the authors supplemented the diet with chitosan oligosaccharides and *Bacillus coagulans*. On the other

hand, our results were several times lower than those reported in (Kadowaki et al., 2013) where the authors used LPS as an immunostimulant. LPS is an endotoxin and it could stimulate the inflammatory response, inducing an increase in the lysozyme activity. The lysozyme activity of SFO-fed fish was comparable with CLO and LO ones but this parameter was lower in SFO+ group compared to CLO+ and LO+ ones. This result indicated that a diet rich in LA had conducted to some alterations in immunostimulation of β-glucan. However, this could be explained by the anti-inflammatory effect induced by the high level of ARA in SFO-fed fish. ARA is the major precursor of highly active eicosanoids (Bell and Sargent, 2003; Wall et al., 2010) that play a role in immune and inflammatory responses (Sargent et al., 2002; Wall et al., 2010), but this LC-PUFA molecule also the precursor of lipoxin metabolism (Chiurchiu et al., 2018). Therefore, the lysozyme level in SFO+ group was comparable with CLO-fed fish but lower than CLO+ and LO+ ones. Besides this, β-glucan is known to be an immunomodulatory factor as cited in the introduction. The β-glucan supplementation in diets rich in ARA (SFO+) could reduce the lysozyme activity compared to diets from the same lipid source but without β-glucans (SFO). A similar explanation can be provided for the lysozyme activity after bacterial challenge.

Alternative complement activity (ACH50), a major pathway of the innate immune response in teleost fish (Yano et al., 1991)' did not show any difference on D63 between fish fed the different lipid sources, regardless of β-glucan supplementation, while significant differences were observed after bacterial infection, as well as a decrease of ACH50 in fish fed diets enriched with β-glucan. The alternative complement pathway is independent of antibodies and is activated directly by foreign microorganisms (Whyte, 2007). Similar results, but with high interspecific variations, were reported in large yellow croaker *Pseudosciaena crocea* (Ai et al., 2007), channel catfish *Ictalurus punctatus* (Welker et al., 2007), rainbow trout (Verlhac et al., 1998) and common carp (Lin et al., 2011; Selvaraj et al., 2009). ACH50 activity was higher in fish fed SFO- and FO-based diets than in those fed a LO-based diet. It has been shown that these fish were richer in ARA and EPA, and these FAs are precursors of the eicosanoid metabolism process, which could enhance the inflammatory response during bacterial infection. Although we did not investigate here the responses of adaptive immune biomarkers, it has been shown in several studies that the dietary supplementation with immunostimulant compounds was able to enhance some adaptive immune responses (Barman et al., 2013; Khuyen et al., 2017; Mo et al., 2015).

Regarding immune gene expression, we found that the effects of dietary lipid sources were only significant for *nk*, whereas dietary β-glucan supplementation significantly influenced the expression of all candidate immune genes. NK cells (knows as cytotoxic cells) are able to eliminate a range of spontaneously xenogeneic targets, traditional targets of natural killer cells in mammals (Hasegawa et al., 1998). NKs are innate lymphoid cells; however, they share a common progenitor with T cells and also directly contribute to adaptive immune responses, interacting with dendritic cells and triggering T cell responses (Parisi et al., 2017), suggesting the influence of NK enhancing factor on the activity of innate and adaptive immune cells. According to (Chan et al., 2009), β-glucan triggers macrophages, neutrophils, monocytes, NK cells and dendritic cells. Our results could confirm this statement as fish fed a diet containing β-glucans displayed up-regulation of *nk*. On the other hand, we observed a down-regulation of *nk* expression in LO and CLO groups compared to the SFO group, this decrease could be

explained by the influence of a diet rich in n-3 PUFA as previous published results in rats (Jeffery et al., 1997) or humans (Kelley et al., 1999; Yamashita et al., 1991) have shown. The other candidate gene, *il8*, was the first known chemokine and pro-inflammatory factor, and plays a key role in the movement of immune effector cells to sites of infection (Kiron, 2012; Zhu et al., 2013). Expression of *il8* has been demonstrated in various teleost species such as rainbow trout (Sigh et al., 2004), common carp (Saeij et al., 2003) and catfish (Chen et al., 2005) in response to infection with pathogens. In our experiment, *il8* gene expression also displayed up-regulation in the CLO+ group and this shows that this immune gene could be stimulated by β-glucans, a kind of fungal polysaccharide. A similar result was reported by (Przybylska-diaz et al., 2013) when they also used β-glucans in an experimental diet.

The highest expression of *pla* and *pge* genes, two key genes in the eicosanoid metabolism process, in SFO-fed fish liver was explained by the abundance of ARA in SFO-fed fish. An up-regulation of these genes could have induced the secretion of ARA from liver membrane layers of fish in the SFO group and eicosanoid metabolism activity was higher here than other groups. A similar result was published for large yellow croaker *Larmichthys crocea* (Lin et al., 2012) in testing the kidney macrophages with different ARA doses. However, the *pla* and *pge* gene expression in SFO+ was lower than SFO-fed fish, indicating the immunomodulatory effect of β-glucans in the diet, which was able to inhibit some inflammatory responses such as prostaglandin production, pain response, etc.

In conclusion, our results have shown that common carp fed plant oils are able to produce substantial amounts of LC-PUFAs for sustaining similar growth rates, immune status and disease resistance to fish fed fish oil. The differences in the capacity for production of LC-PUFA by the two plant oils were associated to differential activation of some immune pathways, which explains how the use of these plant oils did not affect the overall immunocompetence of fish challenged with bacteria. However, the plant oil had induced some alterations of immunostimulatory action of β-glucan and the LA-enriched diet exhibited the over-regulation of genes involved in eicosanoid metabolism in condition without stimulation that may induce to some alterations in fish immune system.

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Chapter 6

A combined *in vivo* and *in vitro* approach to evaluate the influence of linseed oil or sesame oil and their combination on innate immune competence and eicosanoid metabolism processes in common carp (*Cyprinus carpio)*

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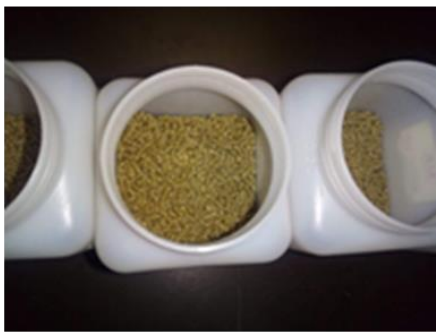

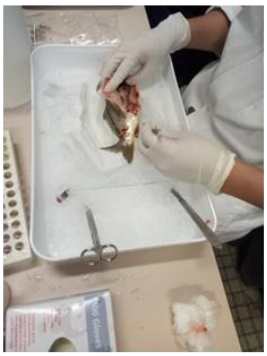

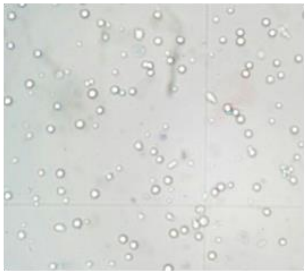


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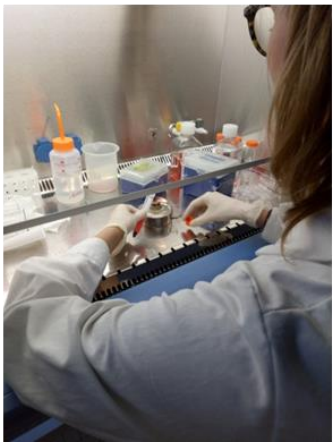


The results obtained from experiments 1 and 2 verified two first hypotheses of our study. The first experiment results have demonstrated that the common carp are able to convert the ARA from n-6 PUFA-enriched plant oil diets (SFO, SO and SSFO); EPA and DHA from n-3 PUFA ones (LO and SLO). The combination between SO and LO (SLO diet) induced the balanced LC-PUFA profile compared to their pure plant oils. The results of experiment 2 have shown the modifications of immune status and immunocompetence of common carp fed different plant oil diets supplemented with β-glucan in basal condition (feeding trial) and bacterial challenge. However, the information focusing on the effects of LC-PUFA amounts in eicosanoid productions was still missing. Therefore, we posed the next scientific question of whether plant oils and their mixture could influence the innate immune competence through modification of eicosanoid metabolism pathways.

The experiment 3 presented in this chapter was performed in order to clarify the hypothesis 3. The details of experimental design are presented in the methodology chapter (Chapter 3). In sum, fish were fed with different lipid sources (cod liver oil, CLO; linseed oil, LO; sesame oil, SO; mixture of sesame oil and linseed oil, SLO) for 6 weeks (nutritional trial). An *in vitro* cell culture was then performed where peripheral blood mononuclear cells and leukocytes isolated from fish in nutritional trial were exposed to LPS for 24h. The results of various immune responses including innate humoral immunity, expression of genes involved in innate and adaptive immune system, and eicosanoid metabolisms in common carp fed plant-derived oils or their blend during a nutritional trial and *in vitro* LPS-exposed leukocytes are presented in this chapter.

Abstract

This study aimed to evaluate the influence of dietary pure linseed oil or sesame oil or a mixture on innate immune competence and eicosanoid metabolism in common carp (*Cyprinus carpio*). Carp of 100.4 ± 4.7 g were fed to satiation twice daily for 6 weeks with four diets prepared from three lipid sources (CLO; LO; SO; SLO). On day 42, plasma was sampled for immune parameter analyses, and kidney and liver tissues were dissected for gene expression analysis. On day 45, HKL and PBMCs from remaining fish were isolated and exposed to *E. coli* LPS at a dose of 10 µg/mL for 24 h. Results show that the SLO diet enhanced feed utilization (P = 0.01), while no negative effects on growth or survival were observed in plant oil-fed fish compared to those fed a fish-oil based diet. Plant oil diets did not alter lysozyme and peroxidase activities or gene expression levels. Moreover, the diets did not affect the expression levels of some genes involved in eicosanoid metabolism processes (*pla, pge2, lox5). Lys* expression in HKL *in vitro* following exposure to LPS was up-regulated in LO-fed fish, while expression levels of *pge2* were higher in SLO fish than in other groups (P<0.05). The highest value for peroxidase activity in HKL exposed to LPS was found in the SLO-fed group (P<0.05). In conclusion, our results indicate that dietary plant oils did not induce any negative effects on fish growth, survival, and immune competence status. Moreover, a dietary combination of SO and LO improved the feed utilization efficiency and seemed more effective in inducing a better immunomodulatory response to LPS through a more active eicosanoid metabolism process.

Keywords: *plant oil; gene expression; lysozyme; complement; peroxidase;*

Abstract abbreviations: CLO: Cod liver oil; LO: Linseed oil; SO: Sesame oil; SLO: blend of sesame oil and linseed oil (v:v 1:1); E.coli LPS: Escherichia coli lipopolysaccharide; HKL: Head kidney leucocyte; PBMC: Peripheral blood mononuclear cell; Lys: lysozyme; pge2: prostaglandin synthase E2

1. Introduction

Fish oil is still the main lipid source in aquatic feed production, and is principally produced from pelagic fish stock such as anchovy, menhaden, and pilchard. This fat source is rich in long chain polyunsaturated fatty acids (LC-PUFA, ≥ 20C) (Durmus, 2018; Nasopoulou and Zabetakis, 2012; Pike and Jackson, 2010), but fish stocks that provide fish oil for aquaculture and other livestock are currently overexploited, and consequently fish oil is very expensive. In contrast, terrestrial plant-derived oils are highly abundant and relatively cheap, and thus could be considered as ideal alternative lipid sources in fish diets. These plant lipid sources naturally lack LC-PUFAs, however some of them are rich in PUFA 18C (Castro et al., 2019; Kutluyer et al., 2017; Mourente and Bell, 2006; Orsavova et al., 2015; Pickova and Morkore, 2007). Moreover, the PUFA profiles of plant oils are not well balanced in relation to fish requirements; consequently, utilization of a blend of plant oils may provide a dietary lipid source that is better balanced in PUFAs (Castro et al., 2016; Kutluyer et al., 2017; M. Nayak et al., 2017b; Teoh and Ng, 2016; Wassef et al., 2015; Xie et al., 2016) in order to satisfy the requirements for precursors of LC-PUFA biosynthesis in fish.

PUFAs can be converted into LC-PUFAs (*e.g.* linoleic acid (LA) to arachidonic acid (ARA); α-linolenic acid (ALA) to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) by biosynthesis processes (Tocher, 2003) but this process is specific (Monroig et al., 2013). Freshwater fish, including omnivorous fish such as common carp, are able to synthesise LC-PUFAs from PUFAs (Nguyen et al., 2019b; Oliva-Teles, 2012; Zupan et al., 2016) and previous studies have shown that the dietary replacement of fish oil by several sources of plant oil - such as sunflower oil, linseed oil, safflower oil, soybean oil, rapeseed oil, and coconut oil - did not lead to negative effects on fish growth or survival in omnivorous fish (Apraku et al., 2017; Ferreira et al., 2015; Hoestenberghe et al., 2013; Peng et al., 2016). Results reported in common carp also demonstrated that dietary lipid sources strongly affected the FA profile of different tissues such as muscle, heart, kidney, intestine, liver, and visceral adipose tissue (Böhm et al., 2014; Ljubojevic et al., 2013; Nguyen et al., 2019b; Qiu et al., 2017; Ren et al., 2012; Schultz et al., 2015; Xu and Kestemont, 2002; Zajic et al., 2016). Nonetheless, information about the influence of dietary lipid sources on the immune response in common carp is still scarce.

The FA composition of cell membrane phospholipids (PLs) in fish depends on dietary lipid sources (Bell et al., 1993; Hulbert et al., 2015; Leray et al., 1986; Mráz et al., 2010; Mraz and Pickova, 2011; Mráz and Pickova, 2009). PLs are the main constituents of cell membranes and their FA composition influences membrane fluidity and cell permeability (Spector and Yorek, 1985); the longer and more unsaturated the carbon chains, the higher the membrane fluidity (Arts and Kohler, 2009; Eldho et al., 2003). There may therefore be a link between n-3 LC-PUFA in the diet and an increase in membrane fluidity (Buda et al., 2006; Kelley et al., 1999; Snyder and Hennessey, 2003). The simplest way to boost the membrane fluidity of fish cells would be to increase the total LC-PUFA content, especially the DHA content in the cell membrane; but the synthesis of such LC-PUFAs may be absent in cases of fish fed terrestrial plant oil-based diets.

The immunomodulatory actions of some compounds might relate to the fluidity of cellular membranes (Maulucci et al., 2016; Mironov et al., 2012; Serrazanetti et al., 2015).

Additionally, LC-PUFAs are released from PL membranes to participate in eicosanoid production by phospholipase (Lall, 2000; Rowley et al., 1995) and this process is involved in the organism's immune defense system (Lall, 2000). LC-PUFAs ≥ 20C, especially ARA and EPA, are the main precursors of eicosanoid metabolism processes (Zhou and Nilsson, 2001). These molecules, including prostaglandins and leukotrienes, play an important role in the fish immune system during inflammatory or other immune responses (Sargent et al., 2002; Wall et al., 2010). On the other hand, some studies have demonstrated that the n-3 LC-PUFAs play a role as anti-inflammatory factors in the immune system (Calder, 2017, 2010; Mullen et al., 2010; Stella et al., 2018; Wall et al., 2010). Consequently, the immune responses reported in fish can be modified depending on the dietary lipid source (Kiron et al., 2011; Mesa-Rodriguez et al., 2018; Montero et al., 2010; Oliva-Teles, 2012; Zhu et al., 2013). However, information is still limited on the influence of LC-PUFA contents on the immunomodulatory ability of some compounds and on the eicosanoid metabolism processes in fish.

The innate immune system of fish, including the cellular and humoral system, helps the animal to defend against infectious organisms and other invaders (Uribe et al., 2011b). One of the most important cell types involved in the immune system is the white blood cells, also called leucocytes, which include lymphocytes, monocytes, neutrophils, eosinophils, and basophils, which seek out and destroy disease-causing organisms or substances (Davis et al., 2008; Ellis, 1977). Leucocytes are produced or stored in many locations in the body, including the thymus, spleen, and other lymphoid tissues (Klosterhoff et al., 2015; Press and Evensen, 1999). The fish immune system, including these cells, can be stimulated by the dietary supplementation of different compounds classified as immunostimulants and this has been shown through *in vivo* experiments (Ai et al., 2007; Anderson and Siwicki, 1994; Bich Hang et al., 2016, 2013; Ibrahem et al., 2010; Khuyen et al., 2017; Mo et al., 2015; Mousavi et al., 2016; Nguyen et al., 2019b; Rodríguez et al., 2009; Selvaraj et al., 2009). Moreover, different fish cells isolated from immune tissues, such as kidney or spleen, have been considered by several authors as *in vitro* models in fish toxicology and immunology (Barman et al., 2013; Cuesta et al., 2003; Pandey, 1994; Reyes-Becerril et al., 2017; Siwicki et al., 1998; Wangkahart et al., 2019). However, there are far fewer studies combining *in vivo* and *in vitro* approaches (Larenas et al., 2003; Lundén and Bylund, 2000) to verify the subsequent effects of vegetables oils on the immune defense of fish.

In this context, the current study was conducted to evaluate if vegetable oils, namely linseed, sesame oils and their mixture in the diets of common carp juveniles would affect their LC-PUFA biosynthesis, immune competence status including immunomodulatory response to an immunostimulant, and eicosanoid metabolism processes. To achieve these objectives, husbandry parameters and various immune functions were tested in common carp juveniles fed pure linseed oil or sesame oil or its blend during a nutritional trial, and the *in vitro* response of its HKL exposed to LPS was examined.

2. Materials and methods

2.1. Fish

Healthy common carp (no disease symptoms or injuries were observed, fish were swimming well, displaying a normal behavior) with an average size of 100.4 ± 4.5 g were collected from a Belgian fish farm (Rue de l'Ile 78, 5580 Lessive, Rochefort, Belgium). Fish were acclimated

in the wet lab of the Research Unit in Environmental and Evolutionary Biology (URBE), Research Institute of Life, Earth and Environment (ILEE), Namur University, Belgium for 2 weeks during which they were fed a mix of all experimental diets.

2.2. Diets

Four experimental diets were formulated from three lipid sources: CLO (cod liver oil, control diet); LO (linseed oil); SO (sesame oil); and SLO, a blend of linseed oil and sesame oil (v/v, 1/1); these plant oils were selected according to their respective contents in LC-PUFAs (Tab. 1).

Table 1. Ingredients and approximate composition of the four experimental diets

	Experimental diets			
	CLO	LO	SO	SLO
Ingredients (g/kg dry matter – DM)				
Soluble fish protein concentrate (SFPC) [a]	270.0	270.0	270.0	270.0
Wheat Gluten [b]	120.0	120.0	120.0	120.0
Gelatin [c]	20.0	20.0	20.0	20.0
Casein [d]	20.0	20.0	20.0	20.0
Starch [e]	395.0	395.0	395.0	395.0
Cod liver oil (CLO) [f]	100.0	0.0	0.0	0.0
Linseed oil (LO) [g]	0.0	100.0	0.0	50.0
Sesame oil (SO) [h]	0.0	0.0	100.0	50.0
Vitamin premix [i]	10.0	10.0	10.0	10.0
Mineral premix [j]	65.0	65.0	65.0	65.0
Total	1000.0	1000.0	1000.0	1000.0
LA (%) [1]	13.6	21.5	42.6	34.2
ALA (%) [2]	1.1	44.1	0.8	19.5
ARA (%) [3]	0.3	--	--	--
EPA (%) [4]	6.3	--	--	--
DHA (%) [5]	9.2	--	--	--
Crude protein, CP (% DM)*	31.5	32.4	32.6	31.0
Crude fat, CF (%)**	11.5	11.9	11.2	11.6
Gross energy, GE (MJ/Kg DM)	18.8	19.1	18.9	18.7
CP/GE (g/MJ)	16.8	16.9	17.2	16.7
CF/GE (g fat/MJ GE)	6.1	6.2	5.9	6.2

Experimental diet nomenclature: CLO: cod liver oil-based diet; LO: linseed oil-based diet; SO: sesame oil-based diet; SLO: blend of sesame and linseed oil-based diet (v/v, 1/1)

[a] *Cook carp concept, 56 Rue de Metz, 57130 Jouy-aux-Arches, France*

[b,c,d,e,f,g,h] *Sigma aldrich, St Louis, MO, USA*

[e] Sigma-Aldrich, St. Louis, MO (USA)

[i] Mineral premix (*to provide g/kg mixture)* was prepared in the laboratory as $CaHPO_4.2H_2O$ = 295.5; $Ca(H_2PO_4)_2.H_2O$ = 217; $NaHCO_3$ = 94.5; KCl = 100; NaCl = 172.4; KI = 0.2; $MgCl_2$ = 63.7; $MgSO_4$ = 34.3; $MnSO_4.4H_2O$ = 2; $FeSO_4.4H_2O$ = 10; $CuSO_4.5H_2O$ = 0.4; $ZnSO_4.7H_2O$ = 10

[j] Vitamin (VTM) premix (*to provide g/kg mixture)* was prepared in the laboratory as Retinyl acetate/VTM A acetate = 0.67; Cholecalciferol/VTM D3 = 0.01; Tocopheryl acetate/VTM E acetate = 34.2; Menadione/VTM K3 = 2.2; Butylated hydroxyanisole/BHA = 1.5; Butylated hydroxytoluene/BHT = 1.5; Ascorbic acid/VTM C = 120; Thiamin/ VTM B1 = 5.6; Riboflavin/ VTM B2 = 12; Pyridoxine/VTM B6 = 4.5; Calcium pantothenate (toxic)/VTM B5 = 14.1; p-aminobenzoic acid/VTM H1 = 40; Cyanocobalamin/VTM B12 = 0.03; Niacin/VTM B3 = 30; Biotin/VTM H, Coenzyme R = 0.1; Choline chloride = 350; Folic acid/VTM M = 1.5; Inositol = 50; Canthaxanthin/E161g = 10

*measured by Kjeldahl method

**measured by Folch method

[1,2,3,4,5] Nguyen et al. (unpublished)

In each diet, soluble fish protein concentrate (SFPC), gluten, casein and gelatin were used as protein sources. The experimental diets were iso-nitrogenous and isolipidic. The formulation

and approximate composition of the experimental diets are presented in Table 1. Ingredients were well mixed and moistened with fresh water (25%) for pelleting to a size of 3 mm. The pellets were then air-dried and stored at 4°C.

2.3. Experimental design

Nutritional trial and *in vitro* assay protocols in our study were conducted in accordance with the European and National legislation for fish welfare, and approved by the local Ethics Committee for Animal Research of the University of Namur, Belgium (Protocol number: UN-KE18/321).

2.3.1. In vivo experiment

Feeding trial

After two weeks of acclimation in a recirculation aquaculture system (RAS), fish were randomly allocated into 12 glass tanks of 100 L (3 tanks per experimental diet) at a density of 14 fish per tank. Fish were fed with the experimental diets to apparent satiation twice a day (10:00 and 16:00) for six weeks. Daily feed intake was recorded to determine feed conversion rate (FCR). During the feeding trial, the rearing condition in the RAS was maintained at a temperature of 20 to 22°C; average dissolved oxygen of 6.5 mg/L; pH of 7.5 and natural photoperiod (Light:Dark 12:12). Nitrite and NH_3/NH_4 were measured once a week and averaged 0.004 and 0.063 mg/L, respectively. The tank system was siphoned daily to remove fish faeces.

Sampling

At the end of the feeding period, on day 42, total fish number and final body weight (FBW) were recorded to determine the survival rate (SR), daily weight gain (DWG), and specific growth rate (SGR). Three fish per tank were randomly anaesthetised with MS222 (120 mg/L, Sigma); blood plasma was individually sampled for lysozyme, alternative complement (ACH50), and peroxidase activities; while the head kidney and liver were dissected for gene expression analyses. The tissue samples were directly frozen in liquid nitrogen and then stored at –80°C.

2.3.2. In vitro experiment

Cell isolation

At day 45 of the feeding trial, head kidney leucocytes (HKL) were isolated from 3 fish from each experimental tank according to a modification of the method described by Braun-Nesje et al. (1982) and peripheral blood mononuclear cells (PBMC) were isolated following Pierrard et al. (2012). Briefly, the HKL were removed from the fish and filtered through a 100 μm nylon mesh (Corning® Cell strainer 100 μm Nylon, Life Sciences) with RPMI 1640 medium (Sigma, USA) containing 1% streptomycin/penicillin (Sigma, USA). HKL were then collected after centrifugation at 800 × *g* and 25°C for 7 min. The fish blood was collected using heparin 0.2% RPMI containing 1% streptomycin/penicillin (Sigma, USA), the PBMC were then isolated using a Ficoll gradient (Ficoll® Paque Plus, Sigma, USA) by centrifugation at 800 × *g* and 25°C for 20 min. The white cell ring containing the PBMC was then collected.

The red blood cells in blood and kidney tissues were removed by lysis buffer (4.14 g NH_4CL + 0.5 g $KHCO_3$ + 0.018 g EDTA in 500 mL MQ water). Both HKL and PBMC were then put in RPMI medium without an antibiotic.

Viability test with LPS

A range doses of LPS concentrations (0; 5; 10; 50; 100; 150 µ/mL) were previously tested for the viability of isolated PBMC and HKL based on the results reported in trout *Oncorhynchus mykiss* (Goetz et al., 2004) and zebrafish *Danio rerio* (Novoa et al., 2009), where the isolated cells were adjusted to 10^7 cells/mL culture medium (RPMI 1640, Sigma, USA); 1% phytohemagglutinin (Gibco™ Phytohemagglutinin, M form, Fisher Scientific); 10% fetal bovine serum (Sigma, USA); 1% HEPES 20 mM (autoclaved solution containing NH_4CL, $KHCO_3$, and EDTA) at 28°C for 24 h of exposure to LPS. The cell viability was determined by a MTS test following the manufacturer's protocol. Briefly, the cells in culture medium (after exposure to different doses of LPS for 24 h in a 96-well plate) was added the MTS test reagent solution (CellTiter 96® Aqueous One Solution Reagent, Sigma, USA); a measurement of absorbance at 490 nm was then carried out after 4 h of incubation at 37°C. The cell viability was calculated by the ratio between the absorbance of the LPS treatment and that of the cell control without LPS. Finally, a LPS dose of 10 µg/mL, which had the highest viability of HKL (83%) and PBMS (98%), was chosen and applied for the *in vitro* trial.

Cell exposure to LPS

HKL and PBMC were isolated on D45 from 3 fish of each tank and adjusted to 10^7 cells/mL of 24-well disk culture medium containing RPMI (Sigma, USA) following the method of Bayne (1986) which was modified for this experiment. The RPMI contained 1% phytohemagglutinin (Gibco™ Phytohemagglutinin, M form, Fisher Scientific); 10% fetal bovine serum (Sigma, USA); 1% HEPES 20 mM (autoclaved solution containing NH_4CL, $KHCO_3$, and EDTA). Cells were exposed to LPS at a dose of 10 µg/mL at 27°C for 24 h. After 24 h of culture, cells were collected by centrifugation at 10000 × *g* at 4°C for gene expression analysis (Table 2), while the medium was used for peroxidase activity analyses.

2.4. Analytical methods

2.4.1. Immune parameter analyses

Lysozyme activity

Lysozyme activity was determined using the protocol of (Ellis, 1990) which was adapted for common carp. Heparin blood plasma (30 µL) was individually suspended in triplicate in 30 µL of PBS buffer (phosphate-buffered saline). A 100 µL bacterial suspension of *Micrococcus lysodeikticus* (Sigma) (200 mg/L in 0.05 M NaH_2PO_4, pH 6.2) was then added to the mix of plasma and PBS buffer. Two readings at 530 nm wave length were carried out with a spectrophotometer after 0.5 and 4.5 min of shaking. The lysozyme activity unit (U/mL) is defined as the amount of enzyme that causes a decrease in absorbance of 0.001/min.

ACH50

The protocol to determine the complement activity was described in (Saha et al., 1993) and adapted for common carp. For this, blood plasma was added by a series of dilutions with Veronal buffer (VCM-F, BioMérieux, Marcy l'Étoile, France) to a 96-well round bottom plate. Wells were then filled with 10 µL of 3% rabbit blood cells (RaRBC, BioMérieux) (70 µL total volume for each well). Samples were incubated at 27°C for 2 h and centrifuged (3000 × *g*, 5 min, 4°C) to collect the supernatant. Then, 35 µL of supernatant was moved to a new 96-well plate and the absorbance was measured at 405 nm. The haemolysin (HLY) was recorded as the highest dilution of plasma showing complete lysis. The ACH50 value was defined as the reciprocal of the plasma dilution which induced 50% haemolysis of RaRBC.

Peroxidase activity

The peroxidase activity assay was inspired by the protocol of (Salinas et al., 2005) and adapted for common carp. Plasma (5 µL) or cell culture medium (20 µL) was added in triplicate into a flat-bottomed 96-well plate, with three wells containing water considered as the blank. To each well was then added HBSS 1× (Gibco, Life Technologies) up to total volume of 75 µL. Then, 25 µL of reaction solution (5 mM H_2O_2, 20 mM TMB, Tetramethylbenzidine dihydrochloride, Sigma) was added to each well and the mixture was incubated at room temperature for exactly 2 min. 25 µl of 4M H_2SO_4 (Sigma) was added at the end of incubation. A spectrophotometer reading at 450 nm was immediately carried out for each well. Peroxidase activity was calculated by the multiplication of the difference between the OD of the sample and that of blank with Df (Df = 1000/sample volume used) and represented by U/mL.

2.4.2. Gene expression analyses

Total RNA of liver, head kidney and HKL was individually extracted from a batch of 3 fish for each tank using 1 mL trizol (Extract-all®, Eurobio, Courtaboeuf, France). The quality of extracted RNA was confirmed using a Nanodrop 2000 spectrophotometer (Thermo Scientific Waltham, MA, USA) and electrophoresis on a 1.2% agarose gel. A pool of 3 RNA samples for each tank was performed to reach a quantity of 12 µg RNA. Pooled samples were treated using a RTS DNAse™ kit (MO BIO Laboratories, Carlsbad, CA, USA) to avoid DNA contamination. The 1 µg of total RNA was reverse-transcripted to cDNA using a RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific). The cDNA sample for each tank was diluted and used for real-time qPCR to determine gene expression. Expression of *lys* (lysozyme), *nkef* (natural killer enhancing factor), *cxc* (chemokine), *il8* (interleukin 8), *b/c2* (classical and alternative complement pathways), *elovl5* (elongase very long delta 5), *fads* (FA desaturase delta 6), *pla* (secreted phospholipase), *pge2* (prostaglandin E2 synthase), and *lox5* (lipooxygenase 5) genes in tissues and in HKL were determined using specific primers. These primers were designed on Primer3 software with the primer quality checked using Amplifix software against sequences of common carp published on Genbank. Primer sequences and gene functions are presented in Table 2. The efficiency of each gene was confirmed before analysis. The *40S* and *18S* genes (Zhang et al., 2016) were used as housekeeping genes. The amplification of cDNA was conducted in triplicate in using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). Thermal cycles and

fluorescence detection were carried out using a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) under the following conditions: 10 min of initial denaturation at 95°C, followed by 40 cycles at 95°C for 30 s and 60°C for 30 s. For analysis, a standard curve of a pool of the cDNA of all samples was included to calculate the PCR efficiency and normalise the transcript levels. The relative levels of RNA were quantified for each gene by densitometry, which was performed by measuring the photostimulated luminescence values using StepOne Software v2.1. Ratios of candidate genes/housekeeping gene products were subsequently calculated for each candidate gene and used to assess the differences in expression levels between experimental groups.

Table 2. Primer sequences for the amplification of genes involved in immune competence, pro-inflammatory response, fatty acid biosynthesis, and eicosanoid metabolism processes in common carp

Genes	Function	Genbank No.	Primer sequence	Efficiency (%)
Immune genes				
il8	Interleukin 8	EU011243	Fw: GTCGCTGCATTGAAACTGAGAG Rv: TTAACCCAGGGTGCAGTAGG	101.1
cxc	Chemokine	AJ550164	Fw: TTGAAACAGAGAGCCAACGCATT Rv: GCTGGTGTTTTGTTGGCAATGA	104
nkef	Natural killer enhancing factor	AB048789	Fw: TGTGATGCCAGATGGACAGT Rv: CCTTGTTTCCGAGGTGTGTT	94.2
lys	Lysozyme activity, C type	AB027305	Fw: GTGTCTGATGTGGCTGTGCT Rv: GAACGCACTCTGTGGGTCTT	103
b/c2	Classical and alternative complement pathways	AB047361	Fw: CAGGCGAATGGGAAATGGAG Rv: GCGTAACATTGTGGCTCTGTTC	106.1
Fatty acid biosynthesis genes				
elovl5	Elongase very long delta 5	KF924199	Fw: CACCAGATCACCTTCCTGCAT Rv: AGCTGCCCTTGAGTGATGTA	105.4
fads	FA desaturase delta 6	AF309557	Fw: CCTCGGACACTATGCTGGAGA Rv: CCCGATTAACAGCGGCTTCA	90.5
Eicosanoid metabolism process genes				
pla	Secreted phospholipase	KF793834	Fw: CTGCATGACAAGTGATGAGCAA Rv: CTGGTGCTCAAATCCATCAGGT	98.9
pge2	Prostaglandin E2 synthase	XM_019098948	Fw: AAGGAATTCATGGGAGGCGATCA Rv: CACACGTCGGTACCAGTTCTTCA	96.7
lox5	Lipooxygenase 5	XM_019066935	Fw: CCCTCCAGCCCAAATTTGAC Rv: ATCCACGCCTGAAGTTCTGA	99.5
Housekeeping genes				
18S	18S ribosomal RNA	FJ710826 (Zang et al., 2016)	Fw: GAGTATGGTTGCAAAGCTGAAAC Rv: AATCTGTCAATCCTTTCCGTGTCC	99.8
40S	40S ribosomal protein	AB012087 (Zang et al., 2016)	Fw: CCCAAGGCCAACAGGGAAA Rv: AGGGCGTAACCCTCGTAGAT	97.8

2.5. Data presentation and statistical analyses

The husbandry parameters of SR, WG, SGR, and FCR were calculated as follows:

SR (%) = 100 × final number of fish/initial number of fish

SGR (%/day) = 100 × (Ln (FBW) – Ln (IBW)) / ΔT

DWG (g/fish/day) = (FBW – IBW) / ΔT

Where FBW and IBW are final and initial body weights respectively and ΔT is the number of days of the feeding trial

FCR = (final biomass – initial biomass + dead biomass)/feed intake

Mean values of all variables were checked for homogeneity by univariate tests (Cochran's), when data were heterogeneous or did not have a normal distribution, a log-transformation of the data was applied and the analysis was performed on the transformed data. Data were then subjected to a one-way analysis of variance (ANOVA 1) for the *in vivo* experiment and two-way analysis of variance (ANOVA 2, with LPS and diet cell type as factors) for the *in vitro* experiment, followed by a *LSD post-hoc* test using the tank replicate as the statistical unit (n = 3). Differences between treatments were considered significant at P value < 0.05. All data were analysed with the statistical package STATISTICA 5.0 (Statsorf, Inc., East 14 Street, Tulsa, USA).

3. Results

3.1. In vivo experiment (feeding trial)

3.1.1. Growth performance

After a 6-week feeding period, FBW averaged 158.4 ± 6.3 g; SR was 100% and FCR ranged from 1.56 to 1.70 (Table 3). No significant differences were observed for growth parameters (WG and SGR) between the different experimental diets. Nonetheless, SO-fed fish displayed the highest FBW, with the lowest in LO fish, and intermediate values were found in other conditions. SLO-fed fish presented the best FCR (P < 0.05), but no significant differences were observed for other fish groups.

Table 3. Husbandry parameters of fish after a 6-week feeding period. Values are presented as means ± SD.

	Diets			
Husbandry parameters	CLO	LO	SO	SLO
IBW	100.6 ± 1.4	98.9 ± 6.9	97.4 ± 4.8	104.5 ± 2.2
FBW	156.2 ± 3.8^{ab}	151.2 ± 3.7^{a}	165.6 ± 1.8^{c}	160.5 ± 4.5^{bc}
WG (%/fish)	55.3 ± 5.9	53.3 ± 9.5	65.0 ± 6.4	58.5 ± 2.7
SGR (%/day)	1.0 ± 0.1	1.0 ± 0.2	1,1 ± 0.1	1.2 ± 0.0
FCR	1.69 ± 0.00^{b}	1.70 ± 0.08^{b}	1.64 ± 0.09^{b}	1.46 ± 0.04^{a}
SR (%)	100	100	100	100

CLO: cod liver oil-based diet; LO: linseed oil-based diet; SO: sesame oil-based diet; SLO: blend of sesame oil and linseed oil-based diet (v/v, 1/1). IBW: initial body weight; FBW: final body weight; WG: weight gain; SGR: specific growth rate; FCR: feed conversion rate; SR: survival rate.

3.1.2. Humoral innate immune response

Data for the activities of the plasma alternative complement (ACH50, from 357 to 425 U/mL), lysozyme (from 46.7 to 78.9 U/mL) and peroxidase (from 101 to 137 U/mL) of common carp on day 42 are summarised in Figures 1a-c. No negative impacts of plant oil-based diets were observed for lysozyme (Figure 1b) and peroxidase activities (Figure 1c), but fish fed SO

displayed a lower ACH50 (Figure 1a) than CLO fish ($P < 0.05$). Amongst the plant-oil diets, lysozyme values were higher in LO-fed fish than in SLO ones ($P < 0.05$).

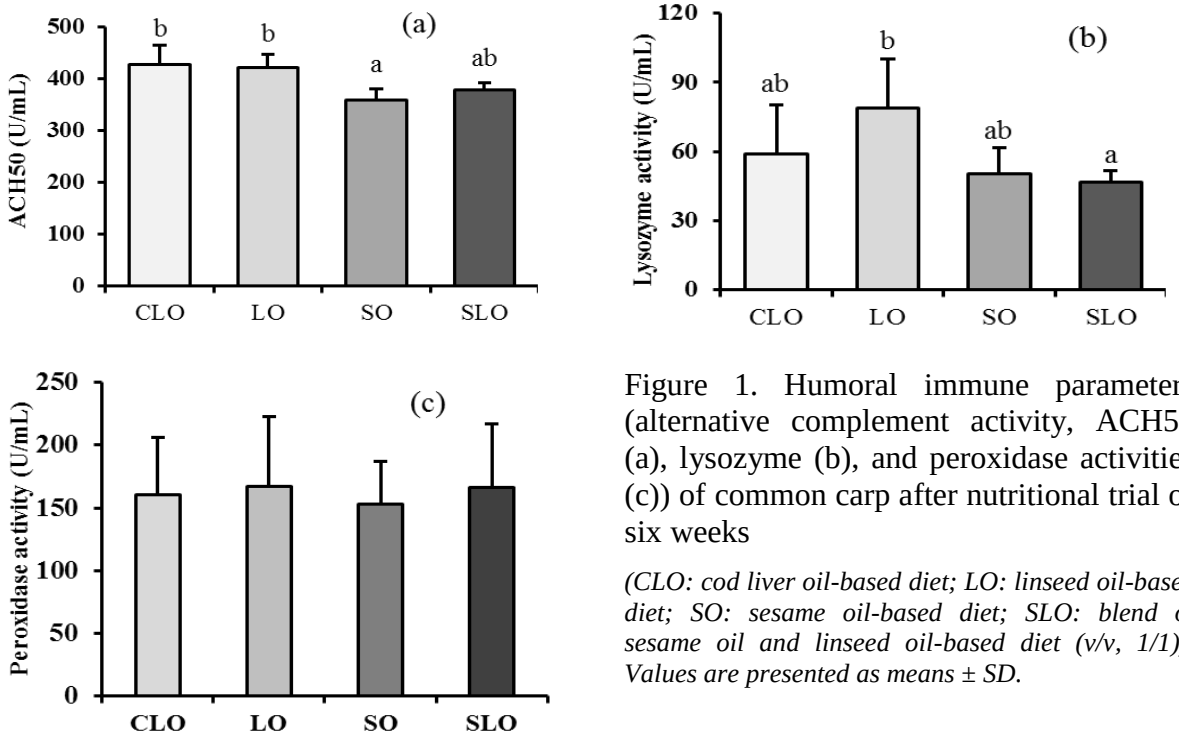
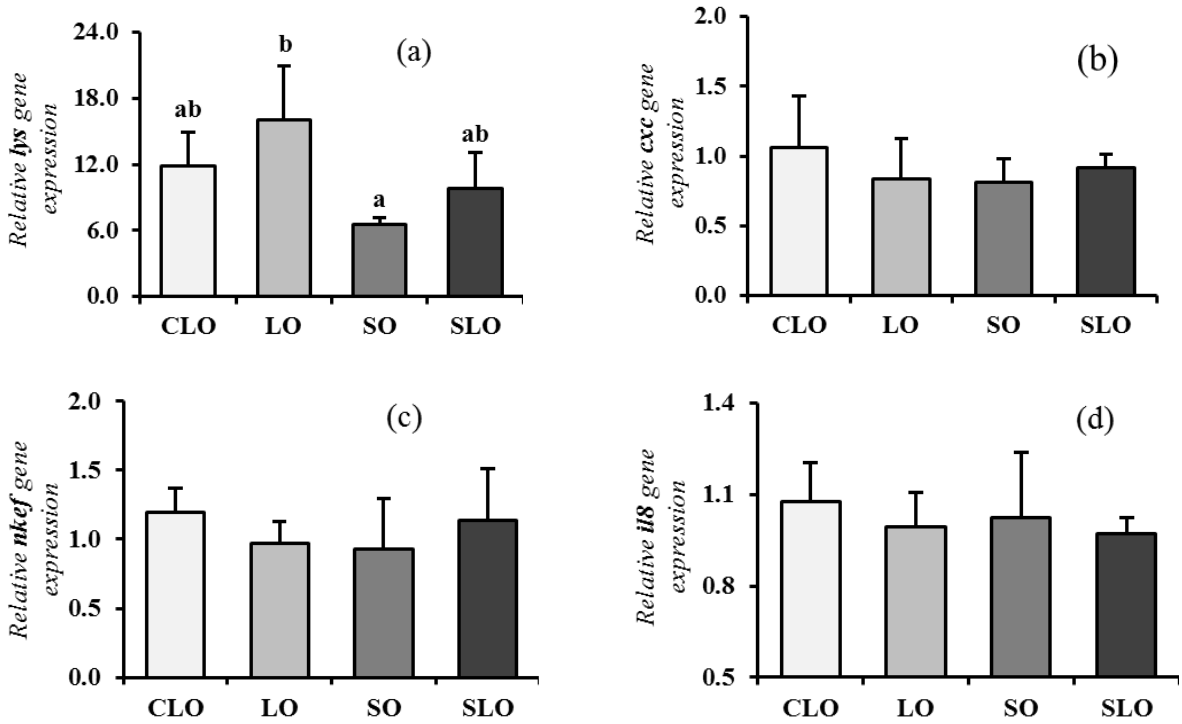


Figure 1. Humoral immune parameters (alternative complement activity, ACH50 (a), lysozyme (b), and peroxidase activities (c)) of common carp after nutritional trial of six weeks

(CLO: cod liver oil-based diet; LO: linseed oil-based diet; SO: sesame oil-based diet; SLO: blend of sesame oil and linseed oil-based diet (v/v, 1/1)). Values are presented as means ± SD.

3.1.3. Relative expression of innate immune genes in head kidney

As for the tested parameters of humoral innate immunity, no negative effects of plant-oil based diets were observed for any of the tested immune genes (Figures 2a-e). Moreover, no significant differences were observed between the tested plant-oil diets, except a lower expression level for the *lys* gene in SO fish than in LO ones (Figure 2a, $P < 0.05$).



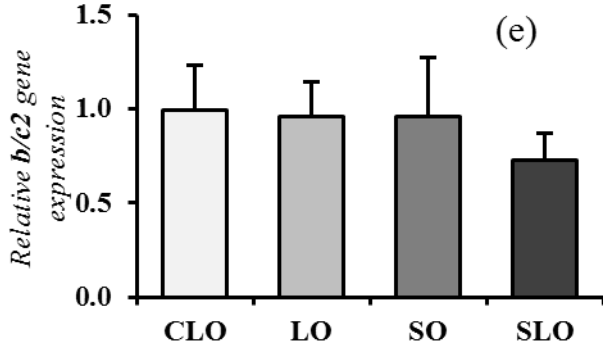


Figure 2. Relative expression of genes involved in immune responses (lysozyme, *lys*, (a); chemokine, *cxc* (b); natural killer enhancing factor, *nkef* (c); inteleukin 8, *il8* (d); and complement activity, *b/c2*, (e)) of common carp after six weeks of feeding with dietary lipid sources.

See Fig. 1 for abbreviations. Values are presented as means ± SD.

Expression of *lys* changed ranging from 6.6 to 16 and strongly vary when compared with other candidate genes *cxc* (fold change of 0.8 to 1.1); *nkef* (0.9 to 1.2); *il8* (about 1.1); and *b/c2* (0.7 to 1.0). The influence of dietary lipid sources was only observed in *lys* gene expression ($P < 0.05$) (Figure 2a) while that of other genes were similar in all experimental groups (Figure 2b to 2e). As for *lys* activity, LO-fed fish presented the highest level of *lys* gene expression and this was two times higher than the lowest group (SO), while intermediate values were found in CLO and SLO groups.

3.1.4. Relative expression of eicosanoid and FA metabolism genes in liver

Expression levels of genes involved in eicosanoid metabolism processes (Figure 3) did not differ significantly between experimental conditions. Accordingly, the dietary lipid source did not influence the relative expression of *pla* (1.1 to 1.7); *pge2* (0.67 to 0.85); and *lox5* (0.88 to 1.83) on day 42.

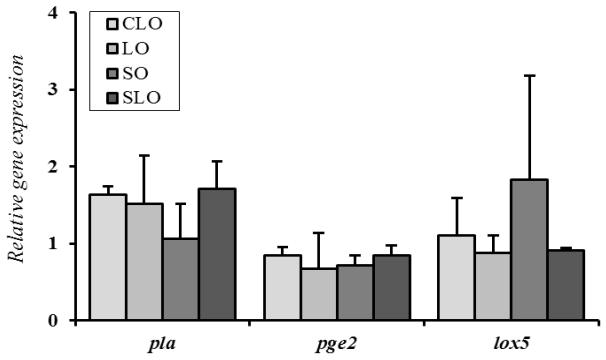


Figure 3. Relative expression of genes involved in the eicosanoid metabolism process in livers of common carp after 6 weeks of feeding with experimental diets.

See Fig. 1 for abbreviations. Pla: Secreted phospholipase; pge2: Prostaglandin E2 synthase, lox5: Lipoxygenase 5). Values are presented as means ± SD

No differences were found in the expression of *elovl5* (1.26 to 1.75) or of *fads* (0.79 to 1.10) on day 42 (Figure 4) between CLO fish and fish fed plant oils or among fish received the three plant oils.

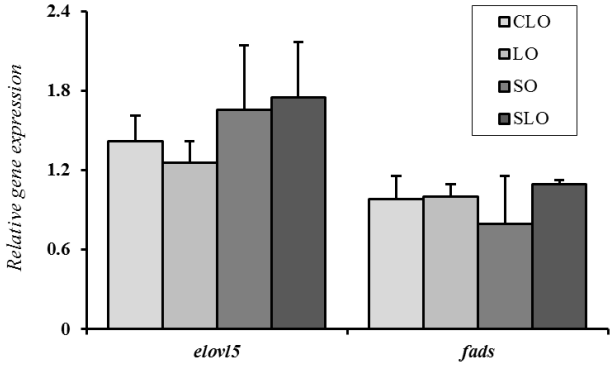


Figure 4. Relative expression of genes involved in FA biosynthesis of common carp at day 42 of the feeding trial.

See Fig 1 for abbreviations. Values are presented as means ± SD

3.2. In vitro experiment

3.2.1. Immune competence of HKL exposed to LPS

Peroxidase activity in the culture medium was analysed for two cell models, however, its level in PBMC (Figure 5.1) was very low compared to that in HKL (Figure 5.2) (6.0 *vs* 147.7 U/mL, respectively) and an influence of LPS treatment as well as dietary lipid sources was only found in HKL.

The peroxidase activity levels of HKL without LPS were higher for the blended SLO group than other plant-oil based groups ($P < 0.05$). Regarding the response to LPS, values of all +LPS groups were higher than those of –LPS groups ($P < 0.05$). Moreover, the highest response was observed in the SLO group ($P < 0.05$), and the values did not differ for other experimental groups.

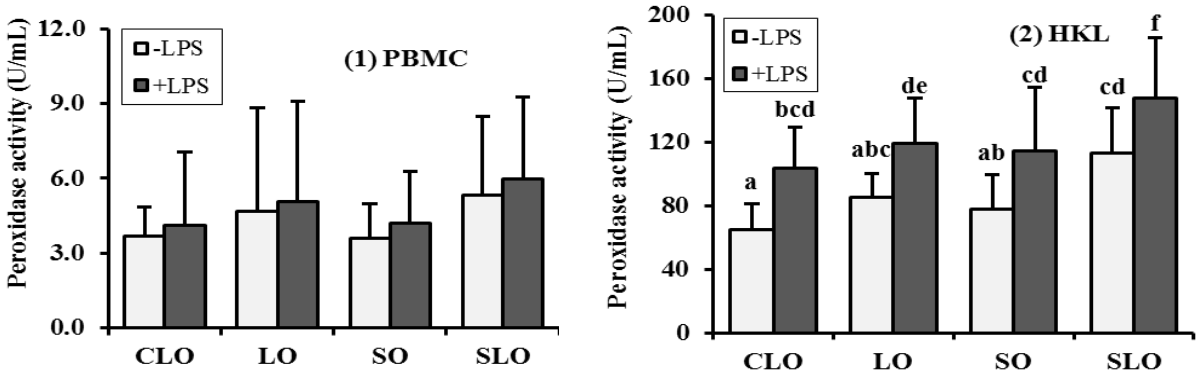


Figure 5. Peroxidase activity in culture medium of common carp PBMC (1) and HKL after 24 h of exposure to *E.coli* lipopolysaccharide (LPS)

Nomenclature of diets: CLO: cod liver oil-based diet; LO: linseed oil-based diet; SO: sesame oil-based diet; SLO: blend of sesame oil and linseed oil-based diet (v/v, 1/1). PBMC: peripheral blood mononuclear cell; HKL: head kidney leucocyte. Data were log-transformed before analysis and values are presented as means ± SD.

3.2.2. Expression levels of genes involved in innate immune functions in HKL isolated from common carp at day 45 of the feeding trial and treated with LPS

Regarding expression levels of genes related to the innate immune response, no significant difference was observed between all the HKL groups without LPS (Figures 6a-e). In contrast, we found an up-regulation in *lys* expression for CLO (+LPS) and LO (+LPS) HKL groups compared to groups without LPS ($P < 0.05$), while those of SO (+LPS) and SLO (+LPS) groups were not up-regulated (Figure 6 a). Precisely, the *lys* expression response to LPS was comparable between CLO HKL groups and LO ones, but was significantly higher ($P < 0.05$) than that of SO and SLO HKL groups. No significant difference in the expression response to LPS was observed for other tested genes (Figures 6b-e).

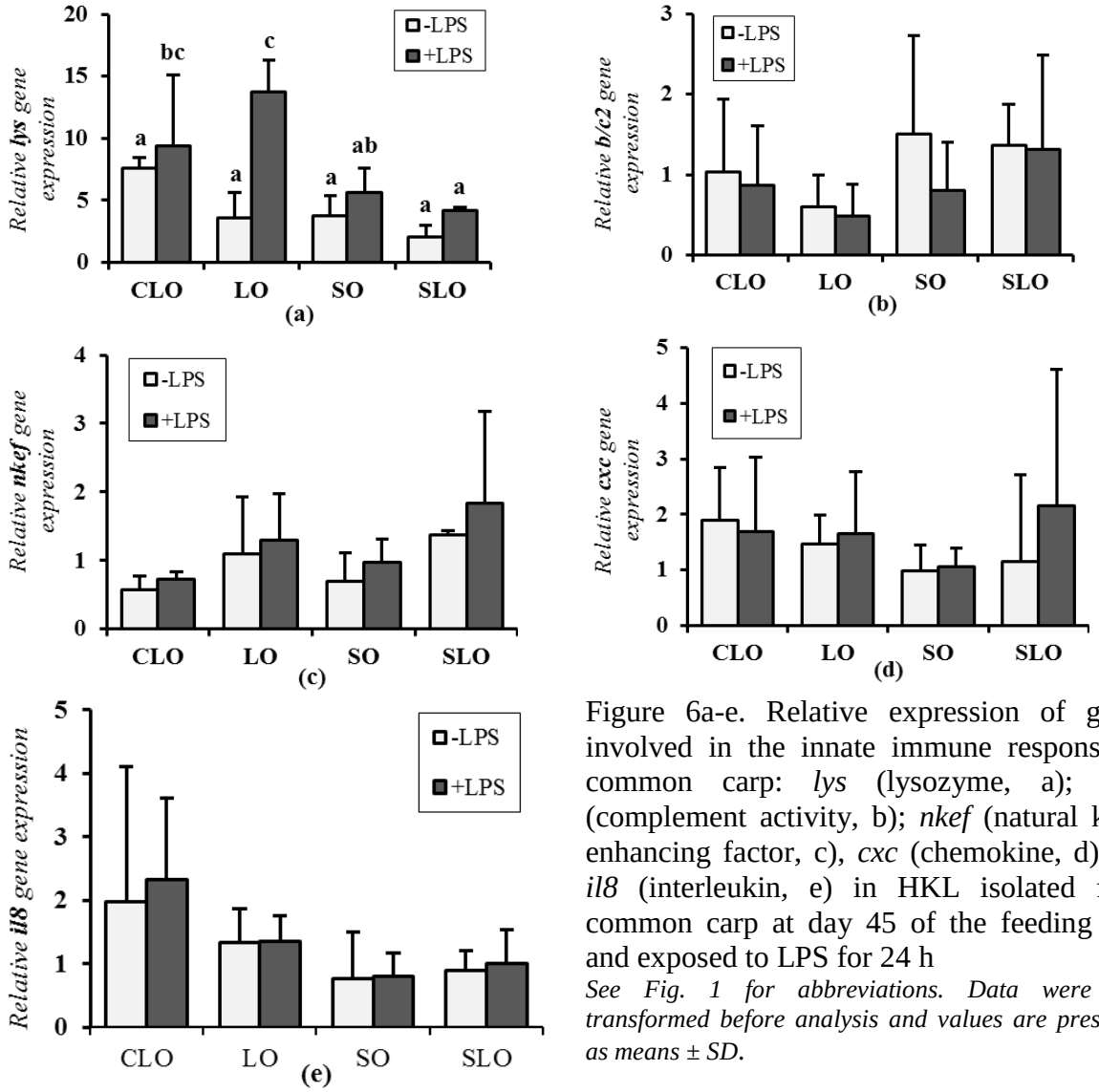


Figure 6a-e. Relative expression of genes involved in the innate immune response of common carp: *lys* (lysozyme, a); *b/c2* (complement activity, b); *nkef* (natural killer enhancing factor, c), *cxc* (chemokine, d) and *il8* (interleukin, e) in HKL isolated from common carp at day 45 of the feeding trial and exposed to LPS for 24 h

See Fig. 1 for abbreviations. Data were log-transformed before analysis and values are presented as means ± SD.

The expression of the *pge* gene was not affected by dietary lipid sources when HKL were not exposed to LPS (Figure 7). In contrast, we observed an up-regulation in *pge2* expression in HKL for SO and SLO +LPS groups ($P < 0.05$) compared to those of –LPS groups, while other experimental HKL groups were not stimulated after 24 h of LPS exposure. Therefore, the *pge* expression response to LPS of HKL from fish fed SO ($P < 0.05$) or SLO ($P < 0.01$) was significantly higher than in HKL from fish fed CLO. Moreover, values of the SLO group were higher than those of pure plant-oil based diets (LO or SO) ($P < 0.01$).

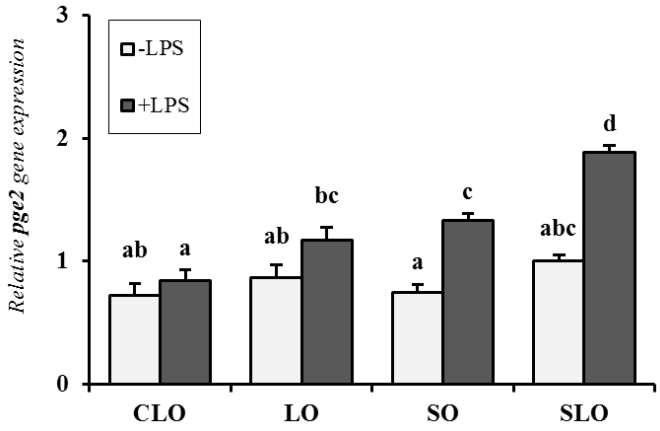


Figure 7. Relative expression levels of the *pge2* gene of HKL isolated from common carp at day 45 of the feeding trial and exposed to LPS for 24 h

See Fig. 1 for abbreviations. Pge2: Prostaglandin E2 synthase). Values are presented as means ± SD

4. Discussion

4.1. Influence of dietary lipid sources on growth, survival and feed utilization

No growth differences were found between the experimental diets, indicating that the plant-derived oil did not negatively influence the fish growth performance. Similar results were recorded in previous studies, as cited in the introduction. The final body weight of SO-fed fish was higher than that of the CLO group. It has been recently demonstrated that ARA supplementation can affect various physiological functions in juvenile yellow catfish (*Pelteobagrus fulvidraco*) and, in turn, influence fish growth through an increase of the availability of circulating glucose and proteins (Ma et al., 2018). In our study, SO diet was rich in LA, precursor of ARA, and we also observed that the tissue composition of fish fed SO diet was rich in ARA (Nguyen et al., unpublished). The SGR values (ranging from 1.0 to 1.2%/day) in this study were similar to those published by Zajic et al. (2016) (about 1.3%/day) and higher than those of Ren et al. (2012) (0.4%/day) working on the same species and in early stages that are supposed to display a higher specific growth rate (juveniles from 40 to 50g). FCR values recorded in our experiment (ranging from 1.5 to 1.7) were lower than those of previous studies using the same species (Yildirim et al., 2013; Zupan et al., 2016), suggesting that this dietary formulation has positive effects on the nutritional status of common carp. The best FCR value was recorded in fish fed a blend of plant-derived oils (SLO diet), indicating that the combination of plant oil sources was able to boost feed utilization in common carp. The mixture of two essential PUFA precursors (LA and ALA) in SLO could provide a suitable FA profile, helping to enhance the feed utilization of common carp. Similar results were shown in the same species (Abbass, 2007) and in other species (El-Tawil et al., 2014). However, the observed improvement of FCR for fish fed SLO diet may not be related only to the FA profiles, as it is the case for growth related parameters. Indeed, in the present study, the expression levels of genes involved in the LC-PUFA biosynthesis did not differ between fish fed CLO and those receiving vegetable oils or amongst fish fed vegetable oils. Moreover, it has been shown that levels of LC-PUFA (namely EPA and DHA) in liver and muscle of carp juveniles were higher in fish fed CLO than in fish receiving LO or its mixture with SO (Nguyen et al., unpublished).

4.2. Influence of dietary lipid sources on the innate immune status and eicosanoid metabolism processes in common carp

4.2.1. Humoral immune status at the end of feeding period

We observed a marked decrease of plasma complement activity by SO, and a trend of increase in lysozyme activity by LO (Figure 1) in comparison to fish oil (CLO), while peroxidase activity level was comparable between fish groups at the end of the feeding trial. These results may indicate that only SO may affect some functions of the innate immune system of common carp. The interaction of nutrition and the immune system in fish has long been known, but this relationship is complex (Siwicki et al., 2006). Previous studies on this aspect have shown the influence of dietary lipid source on the immune response in fish due to the dietary FA profiles, but information on the effects of individual FAs is still limited (Kiron, 2012). Diets rich in ALA could improve immune competence and disease resistance (Chen et al., 2016; Cornet et al., 2018; Geay et al., 2015b) but a diet rich in LA was also reported to enhance the immune response in fish (Ferreira et al., 2015; Makol et al., 2009). Moreover, the balance between n-3

and n-6 LC-PUFAs might create the most favorable immune response and the dietary n3/n6 ratio should be close to 1 (Bhardwaj et al., 2016; Gómez Candela et al., 2011; Simopoulos, 1991). Previous studies on salmonids and other freshwater fish showed that FA composition in fish was especially rich in n-6 LC-PUFA when fish were fed high levels of dietary n-6 PUFAs such as sunflower oil (Zuo et al., 2015a), rapeseed oil (Montero et al., 2003), soybean oil, safflower oil, peanut oil (Sagne et al., 2013), or LA (Cornet et al., 2018), while the n-3 LC-PUFAs were abundant in fish fed dietary lipid sources rich in n-3 PUFA such as linseed oil (Ferreira et al., 2015; Montero et al., 2003; Xu and Kestemont, 2002; Zuo et al., 2015a) or ALA. Moreover, the n-3/n-6 ratio was reported to be close to 1 as found in linseed oil based diets Nguyen et al., unpublished). The latter information could explain why, in our study, alternative complement activity in CLO, LO, and SLO-fed fish was higher than in SO-fed fish, and lysozyme activity in LO-fed fish was higher than in SO-fed fish.

Lysozyme is a bacteriolytic enzyme that is widely distributed throughout the body and is part of nonspecific defense mechanisms in most animals (Uribe et al., 2011b). Besides an antibacterial function, it promotes phagocytosis by directly activating polymorphonuclear leucocytes and macrophages, or indirectly by an opsonic effect (Saurabh and Sahoo, 2008). Globally, the plasma lysozyme activity in common carp is low, about 100 U/mL (J. L. Wang et al., 2015; Wang et al., 2006; Wu et al., 2007; Yin et al., 1995) compared to other species such as tilapia *Oreochromis mossambicus* (ranging from 770–1000 U/mL; Christybapita et al., 2007), rainbow trout *Oncorhynchus mykiss* (from 600–1000 U/mL, Verlhac et al., 1996); Atlantic salmon *Salmo salar* (2050 U/mL, Lie et al., 1989). The highest lysozyme activity was measured in the plasma of LO-fed fish (78.9 U/mL), more than two times higher than the values reported by Lin et al. (2011) or those reported by Lin et al. (2012) (about 40 U/mL) in *Cyprinus carpio Koi.* Alternative complement activity (ACH50) was also influenced by dietary lipid sources and this immune parameter was comparable in plant oil-fed fish and fish oil-fed fish. Similar results, but with high interspecific variations, were reported in European seabream *Sparus aurata* (Montero et al., 2003) and Nile tilapia *Oreochromis niloticus* (Yildirim-Aksoy et al., 2007).

4.2.2. Innate immune gene expression and eicosanoid genes at day 42 of nutritional trial

Regarding the effect of plant oils on other immune relays, similar results compared to results of humoral immune parameters were observed for expression levels of the studied innate immune genes in head kidney. Indeed, relative *lys* expression in LO-fed fish in head kidney was higher than in SO-fed fish, and similar to other experimental conditions (Figure 2). We did not find any difference between experimental groups for the expression of the two genes involved in pro-inflammatory (*cxc, il8*) or other innate immune responses (*b/c2 and nkef*). The chemokines *cxc* and *il8* tested in our case, are pro-inflammatory cytokines that can be induced during an immune response to promote the migration of immune cells to a site of infection by binding to and activating chemokine receptors (Fernandez and Lolis, 2002), its expression is increased following infection with the bacterial component (Tanekhy et al., 2009). Therefore, the expression of genes involved in chemokine production in our study did not differ between experimental groups in the condition without bacterial infection. The complement is also activated either directly by microorganisms or by antibody-antigen (Ag-Ig) complexes (Holland and Lambris, 2002). Thus, no differences were observed for *b/c2* gene expression even if AHC50 was influenced by the dietary SO at the end of the feeding period.

All the results obtained concerning the studied innate immune genes indicated no marked negative effects of plant oils on various immune functions, namely bactericidal and pro-inflammatory processes.

The explanation about the lack of marked effect of plant oils on various parameters of the innate immune system in the present study can apply for *pge2* and *lox5* on day 42 of the feeding trial. Prostaglandins and leukotriene, the active eicosanoids participating in the inflammatory response (Wall et al., 2010) induce the expression of these genes that do not increase under normal conditions. The same observation was found in zebrafish for eicosanoid metabolism genes such as cyclooxygenase 1, cyclooxygenase 2 and prostaglandin E2 before bacterial infection (Nayak et al., 2018).

4.3. Influence of dietary lipid sources on the innate immune competence and eicosanoid metabolic processes of carp HKL exposed to LPS

The tested plant oils did not negatively affect the innate immune status and the immune response to LPS of carp leucocytes in terms of myeloperoxidase (MPO) activity and expression of innate immune genes. MPO is an important enzyme involved in the defense against bacterial and fungal infection. MPO is produced by leucocytes, principally in neutrophils (Lin and Austin, 2002) and also in monocytes though at lower levels (Davies, 2011). MPO has a greater impact in inflammatory conditions (Klimiuk et al., 2006) than normal conditions. This could explain why peroxidase activity on day 42 of the feeding trial did not differ between the experimental groups. As the PBMC included monocytes and lymphocytes but without granulocytes, including neutrophils, basophils and eosinophils (Kleiveland, 2015), peroxidase activity in HKL was found to be several times higher (147.7 U/mL) than in PBMC (5.98 U/mL) suggesting the utilization of the HKL for inflammatory pattern instead of PBMC in humoral innate immune response studies. The highest value of peroxidase activity was observed in SLO HKL (147.7 U/mL) and other groups were similar (Figure 5). This could be explained by the abundance of both ARA and EPA and more balanced in PUFA precursors of this experimental oil. In SLO fish, ARA level was higher than CLO and LO group while EPA level was higher than SO fish; besides, the LA and ALA levels in SLO diet or fish displayed the intermediate values compared to LO and SO groups. The eicosanoids include prostaglandins and leukotrienes (produced from ARA, EPA) and are one of the main pro-inflammatory mediators (Sargent et al., 2002; Wall et al., 2010). High levels of prostaglandin or related gene expression have been reported in fish fed dietary lipid sources rich in LA or ARA (Asturiano et al., 2000; Bell et al., 1993; Tian et al., 2016). Therefore, the highest value of peroxidase activity obtained in SLO group could be explained by these arguments. In our study, we also observed the highest level of *pge2* expression in the SLO (+LPS) group not in the SLO (-LPS) group. Peroxidase activity was comparable with that in plasma after the nutritional trial (167.3 U/mL) indicating that the peroxidase enzyme of common carp was principally produced by neutrophils in the head kidney and these were strongly stimulated by an immunostimulant compound. Similar results, but in head kidney tissue, were observed in *Labeo rohita* after $ZnCl_2$ treatment (Mushtaq et al., 2017).

Regarding the response of the tested innate immune genes, chemokine gene expressions, *cxc* and *il8*, in HKL were not stimulated by LPS after 24 h of exposure. The *cxc* and *il8* are cytokines that can activate eicosanoid production (Dudzinski and Serhan, 2004). The expressions of *il8* in grass carp *Ctenopharyngodon idella* HKL (Wu et al., 2012) or of *cxc* in

common carp (Gonzalez et al., 2007) were reported to be higher at the early stage of the inflammatory process (< 12 h). This explanation could apply to our results, although the *pge2* in HKL presented an up-regulation in the +LPS group after 24 h, the expression of these chemokine genes did not differ between –LPS and +LPS groups. We observed an up-regulation of *pge2* expression in HKL isolated from fish fed a diet rich in LA (SLO and SO fish) and also in ALA (LO fish), suggesting that in the condition stimulated by the antigen (or immunostimulant) the HKL prioritised the biosynthesis of ARA, the precursor to eicosanoids in the inflammatory response.

The stimulating ability might also link to cell membrane permeability which is influenced by PL composition as previously mentioned. In fish, previous studies have demonstrated the effects of dietary FAs on the modification of membrane PL (Bell et al., 1993) or the FA profile of tissues (Ma et al., 2018; Mellery et al., 2017; Teoh and Ng, 2016). ARA, EPA, and DHA increase the permeability of cell membranes (Yang et al., 2011). However, when membrane concentration of ARA is higher than EPA and DHA leading to a decrease in membrane fluidity (Husted and Bouzinova, 2016) and consequently, LPS absorption. We hypothesise that the amount of LPS absorbed in HKL of CLO and LO-fed fish (rich in EPA and DHA) was higher than in SO and SLO-fed fish (rich in ARA), inducing a higher *lys* expression in the former experimental conditions. In addition, although a low concentration of prostaglandin E2 is required for normal immune function, high concentrations are immunosuppressive (Bell and Sargent, 2003).

In conclusion, the results confirm that the use of plant oils in the common carp diet did not induce any negative effects on fish growth and fish survival. A combination of plant-derived oil rich in LA and ALA may enhance the feed efficiency. The innate immune status of common carp fed the plant oil-based diets was comparable to that of fish fed the fish oil-based diet, except a decrease in complement activity in fish fed SO diet. Levels of peroxidase activity and gene expression of prostaglandin E2 were enhanced in HKL from fish fed diets SLO when stimulated by LPS indicating that this mixture of plants oils sustained as well a good immune defense in common carp. Together, *in vitro* combined with *in vivo* approaches help to better demonstrate that pure linseed oil or its mixture with sesame oil has no negative influence on growth related parameters and innate immune competence of common carp.

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Chapter 8

General discussion

1. Common carp as an ideal fish in researches on the influences of lipid sources on FA biosynthesis and immunology

The common carp is an important aquaculture species; it is the most cultured fish for human food consumption. In research, this species is an important fish for a wide range of studies focusing on physiology, such as nutrition and farming conditions (Billard, 1999), fish diseases and immunology (Adamek et al., 2018; Ardó et al., 2010; Behrmann-Godel, 2015; Gómez Candela et al., 2011; Vera-jimenez et al., 2013; Zhang et al., 2011) and fish flesh quality (Böhm et al., 2014; Schultz et al., 2015; Zajic et al., 2016). When compared with zebrafish, studies on common carp can benefit from the large body size of the species, providing sufficient organ material source for various physiological and molecular analyses (Henkel et al., 2012).

1.1. Availability of validated immune parameters in common carp

In this study, almost all humoral immune parameters including innate immune (lysozyme, alternative complement, peroxidase activity, nitric oxide level) or adaptive immune (total Ig) variables could be analyzed in common carp plasma as well as in lymphocyte culture medium. These immune variables were also investigated to assess the immune responses in previous studies (Gopalakannan and Arul, 2006; Kadowaki et al., 2013; Lin et al., 2012, 2011; Nguyen et al., 2016; Pionnier et al., 2013; Sakai et al., 2001; Selvaraj et al., 2009; Tassakka and Sakai, 2002). Moreover, other studies also used some other immune parameters involved in humoral or cellular immune system such as nitroblue tetrazolium (NBT), phagocytic activity, leucocyte count, specific antibody, bactericidal activity and superoxide dismutase (Falco et al., 2012; Harikrishnan et al., 2005, 2010; Huttenhuis et al., 2006; Kadowaki et al., 2013; Lin et al., 2012, 2011; Nguyen et al., 2016; Sakai et al., 2001; Selvaraj et al., 2005; Tassakka and Sakai, 2002; Vera-jimenez et al., 2013; Watanuki et al., 2006).

1.2. Confirmation of LC-PUFA biosyntheses from PUFA precursors in common carp

The freshwater/omnivorous fish are able to biosynthesize the LC-PUFAs from PUFA precursors by a series of elongation and desaturation reactions (Oliva-Teles, 2012). Previous studies in common carp reported that the utilization of plant oil sources rich in PUFAs, such as linseed oil, corn oil, rapeseed oil or a blend of plant oils, supported good levels of LC-PUFAs associated with higher expression levels of genes involved in FA metabolism, compared to those of fish fed a fish oil-based diet (Ljubojević et al., 2015; Mráz et al., 2010; Mraz and Pickova, 2011; Ren et al., 2015, 2012; Schultz et al., 2015; Trbović et al., 2013; Zajic et al., 2016). This was also recorded in our study (experiments 1 and 2). High contents in EPA and DHA in carp liver and muscle were found in fish fed diets rich in ALA. Accordingly, the ARA content in carp tissues increased with the increase of dietary LA content and the ARA content in liver of fish fed on the SFO, SO, and SSFO diets was even higher than that of fish fed on the CLO diet. These results confirmed the good expected capacity of LC-PUFA conversion from PUFA precursors. These results also suggest that common carp is an ideal species to assess the plant oil sources that could be used in fish diets.

1.3. Stimulation of immune system of common carp by immunostimulant compounds

The fish immune system can be stimulated by an immunostimulant and that was also demonstrated in common carp. Previous researches reported that immune parameters such as

lysozyme, complement, macrophage activity or the expression of genes involved in the immune system could be stimulated by an immunostimulant supplementation, such as β-glucan, lipopolysaccharide (LPS), nucleotides from yeast RNA, chitosan or plant extracts by injection, oral administration, or immersion (Herczeg et al., 2017; Kadowaki et al., 2013; Kono et al., 2004; Lin et al., 2012; Nguyen et al., 2016; Pionnier et al., 2013; Przybylska-diaz et al., 2013; Sakai et al., 2001; Watanuki et al., 2006). In our study, the immune system of common carp was also stimulated by such immunostimulant compounds (β-glucan and LPS). These stimulations were seen through the increase of several target immune variable levels (peroxidase and lysozyme activity, nitric oxide and total Ig level) and expressions of marked immune genes (*il-1, il-6, il-8, tnf-α, inos, pge2*). Based on these results, we showed that the common carp can be a fish model to test the immune stimulating capacity of different candidate immunostimulants.

1.4. Utilization of leukocytes isolated from common carp as cell model in in vitro approaches

The viability of lymphocytes (PBMC and HKL) isolated from the common carp was high (about 95% after 24h of culture) under *in vitro* conditions. This result was comparable to those of cells isolated from rainbow trout (Crippen et al., 2001; Leblond et al., 2001; J. Wang et al., 2019). In the LPS-exposure, HKLs exhibited the stimulation via the increase of target humoral immune parameters (peroxidase activity in experiment 3; NO and total Ig level in experiment 4) as well as through the up-regulation of several pro-inflammatory mediators such as *inos*, cytokines/chemokines (*tnf-α, il-1, il-6, il-8, cxc*) and eicosanoid metabolism process (*pge2*) (experiments 3 and 4). These observations suggest that the use of this fish cell source is suitable for immune researches.

1.5. Availability of gene sequences for physiological researches

The number of genes used in this study was sufficient to establish the metabolism pathways in nutrition, immunology and its interaction in common carp. For fatty acid biosynthesis, the *fads* and *elovl5* are two key genes in desaturase and elongase processes of fatty acid bioconversion. The full or partial sequence of these genes is published on gene bank and the results available in other studies also demonstrated the activity of these genes in fish fed different lipid sources (Ren et al., 2015, 2012). In the immune system, a wide range of genes were validated and helped to explain the influence of experimental factors including innate (*lys, b/c2, il-8, cxc, il-1, il-6, tnf-α, inos, nf-kb, prdx-3, gpx-1, tlr-4, il-10, tgf-β1, nf-kbi*) and adaptive immune system (*nkef*). Especially, the key genes to demonstrate the influence of dietary lipid on the immune responses were available including eicosanoid metabolism genes (*pla2, pge2* and *5-lox*). However, the sequences of some important genes involved in the metabolism of anti-inflammatory lipid mediators (resolvins and lipoxin) are not yet available for common carp in gene bank.

2. Influences of fish oil substitution by plant oils on fish performance in common carp

The common carp is an omnivorous fish and, as most of these fish species, is able to use the plant-derived oil without negative effects on fish growth, feed utilization and survival (Oliva-Teles, 2012). These observations were reported in previous studies in the same species or in others (Carmona-Osalde et al., 2015; Mellery et al., 2017; Nguyen et al., 2019b; Peng et al., 2016; Thanuthong et al., 2011; Turchini et al., 2011).

No significant reduction of the husbandry performances was recorded in plant oil-fed fish compared to those fed fish oil in all experiments. Moreover, fish fed SFO (experiment 2) and

SO (experiment 3) diets displayed higher final body weight than the one of fish fed CLO diet. These observations have demonstrated the possibility of a fish oil replacement by plant-derived oils in carp diet, as far as growth is concerned. In the present study, the experimental diets were prepared from similar protein and carbohydrate sources. Therefore, the observed differences may be only influenced by the oil sources. The CLO diet did not support the best growth performance in common carp, suggesting that, for this species, the lipid composition is not a strict limiting factor for an optimal growth. This has been previously reported for the same species by Ren et al. (2012), Yildirim et al. (2013) and Nguyen et al. (2019b, 2019a), as well as for Arctic charr (*Salvelinus alpinus*) by Tocher et al. (2006), halibut *Hippoglossus hippoglossus* by Haugen et al. (2006), rainbow trout *Oncorhynchus mykiss* by Thanuthong et al. (2011) and African catfish *Clarias gariepinus* by Sourabié et al. (2018).

In the first experiment, fish growth was low when compared to previous studies on the same species (Abbass, 2007; Yesilayer et al., 2011; Yildirim et al., 2013). This lower growth could probably be attributed to the difference in dietary protein sources. Almost all previous studies used industrial fish meal as the main protein source. On the contrary, in our experiment, casein, gelatin and wheat gluten were used and no fish meal was included due to its fish oil content ranging from 5 to 10 % (Jensen et al., 1990). A dietary fish meal inclusion would have led to a "passive fish oil supplementation", which could have modified the results on fatty acid composition of fish. To establish an extreme experimental condition, we formulated the diet by totally replacing the fish oil by plant oil. However, the substitution of fish meal by plant-based ingredients usually induces a reduction of growth in fish. The study of Ren et al. (2012) with common carp fed on a diet formulated with casein and gelatine showed relatively low growth rate, similar to the one obtained in our study. Similar results of fish growth were also reported for the zebrafish *Danio rerio* fed on casein as unique dietary protein source (Smith et al., 2013) and for the goldfish *Carassius auratus* fed on a diet made with a plant-based protein source (Bilen and Bilen, 2013). Alternative sources of protein, such as the soluble fish protein concentrate (SFPC), could have been used in order to support a higher growth performance, while using plant-derived oils. This fish meal source was applied in the latter experiments (experiments 2, 3, 4) and the results showed a significantly better growth in experimental fish. The SGR values in the experiment 1 ranged from 0.5 to 0.7%/day (Chapter 4) and this value was improved in the next experiments using SFPC as protein source (ranging from 1.0 to 1.3; 1.0 to 1.2 and 1.4 to 1.6 %/day in experiments 2, 3 and 4, respectively). Our results were similar to those of the study of Zajic et al. (2016) (about 1.3%/day) and higher than those reported by Ren et al. (2012) (0.4%/day) for the same species.

We also observed that supplementation with an immunostimulant (β-glucan) did not improve the husbandry parameters. Similar observations were found in previous studies with common carp where the authors used different compounds such as β-glucan (Selvaraj et al., 2009), chitosan (Lin et al., 2011) or May chang *Litsea cubeba* leaf powder (Nguyen et al., 2016) as dietary immunostimulants.

The feed utilization capacity (FCR) recorded in the experiments (ranging from 1.8 to 2.2; 1.5 to 1.7 and 1.6 to 1.9 in experiments 2, 3, 4, respectively) were similar or lower than those of some studies in the same species (Yildirim et al., 2013; Zupan et al., 2016), suggesting that this dietary formulation has positive effects on the nutritional status of common carp. The best FCR value was recorded in fish fed a blend of plant-derived oils (SLO diet, experiment 3), indicating

that the combination of plant oil sources was able to boost the feed utilization in common carp. This result could be explained by the fact that the mixture of two essential PUFA precursors (LA and ALA) in SLO could provide a suitable FA profile, enhancing the feed utilization in common carp. Similar results were shown in the same species (Abbass, 2007) and in other species (El-Tawil et al., 2014) using a blend of terrestrial vegetable oils in diet.

Survival rate recorded in this study was high (ranging from 90 to 100%), and did not significantly differ between the different fish groups, indicating that the total replacement of fish oil by plant oils did not induce any negative effect on common carp survival.

3. Influences of plant oil utilization on fatty acid composition of common carp

3.1. Influence of fatty acid compositions in oil sources on lipid digestibility

High concentrations of both C16:0 and C18:0 in dietary lipids from animal origin have been reported to exert a negative impact on the dry matter and lipid digestibility of fish (Caballero et al., 2002; Menoyo et al., 2003). In this study, both C16:0 and C18:0 amounts in the SFO diet were reduced as compared to the other experimental diets. This may explain the higher apparent digestibility coefficient (ADC) values for this diet than for the LO diet, while the SO diet, rich in both saturated fatty acid, exhibited the lowest lipid ADC value. The intake of digestible ALA was higher with the LO and SLO diets while that of digestible LA was higher with the SFO, SO, SLO and SSFO diets. These PUFAs were at their lowest levels in the CLO diet as compared with the plant-derived oil diets. Differences in lipid digestibility as those highlighted in the present study should be taken into account in feed formulation for carp. If a lipid source with a low lipid ADC is used, it is recommended to supply a higher dietary lipid level than the one classically required by common carp. This supplementation could compensate for the low lipid digestibility and could even potentially increase the fatty acid amount in tissues. On the contrary, the dietary lipid quantity may be reduced in case of a high lipid ADC value.

3.2. Dependence of fish tissue FA composition on FA profiles of dietary lipid sources

FA profiles in common carp tissues reflected those of their respective diets. In this study, the FA composition of common carp liver and muscle was significantly affected by the dietary FA composition. Tissues from fish fed CLO diet were rich in EPA and DHA while those from LO-fed fish were rich in ALA, and SFO-fed fish were rich in LA. The fish fed the mixture of two plant oils (SLO) exhibited the intermediate value in both PUFA precursors compared to pure plant oil-fed fish. This FA store could provide a balanced profile in PUFA precursors in LC-PUFA bioconversion. These observations were also reported in the same or other species (Geay et al., 2015b; Montero et al., 2010; M. Nayak et al., 2017a; Nguyen et al., 2019b; Thanuthong et al., 2011; Torrecillas et al., 2017; Xu and Kestemont, 2002; Zupan et al., 2016).

Previous results demonstrated that dietary lipid sources strongly affect the FA profile of different tissues such as muscle, heart, kidney, intestine, liver, brain and visceral adipose tissue and the main target tissues are liver and muscle (Böhm et al., 2014; Geay et al., 2015b; Ljubojevic et al., 2013; Montero et al., 2010; Nguyen et al., 2019b; Qiu et al., 2017; Ren et al., 2012; Schultz et al., 2015; Thanuthong et al., 2011; Turchini et al., 2011; Xu and Kestemont, 2002; Zajic et al., 2016). The FA composition of cell membrane phospholipids (PLs) in fish is also reported to be dependent on dietary lipid sources (Bell et al., 1993; Hulbert et al., 2015; Leray et al., 1986; Mráz et al., 2010; Mraz and Pickova, 2011; Mráz and Pickova, 2009). PLs

are the main constituents of cell membranes and their FA composition influences membrane fluidity, cell permeability (Spector and Yorek, 1985), and immune system by the release of LC-PUFA from PL membrane in the inflammatory responses (Calder, 2017; Chiurchiu et al., 2018; Medzhitov, 2008; Medzhitov, 2008). These LC-PUFAs participate to the immune system and play a role as a lipid mediator in pro- and anti-inflammatory responses (Calder, 2017, 2010; Chandrasekharan and Sharma-Wali, 2015; Chiurchiu et al., 2018; Medzhitov, 2008; Mullen et al., 2010; Sargent et al., 2002; Stella et al., 2018; Wall et al., 2010). Consequently, there is a strict interaction between the dietary FA and immune responses in fish.

3.3. Conversion ability of LC-PUFAs from PUFA precursors in common carp

The abundance of ALA in the LO and SLO diets led to a relatively high content in EPA and DHA in carp muscle while the ARA content increased with the increase of dietary LA content; moreover, the ARA content in tissues of fish fed SFO, SO and SSFO diets was even higher than that CLO-fed fish. This suggests a good ability of common carp to biosynthesize ARA from LA and EPA, DHA from ALA. The similar trend was also noticed in previous studies in carp (Nguyen et al., 2019b; Ren et al., 2012; Zupan et al., 2016). The EPA and DHA contents in our study are higher than those reported in previous studies on common carp, such as those of Stancheva and Merdzhanova (2011); Ljubojevic et al. (2013) and Župan et al. (2016). Interestingly, the carp muscle EPA and DHA contents found in the LO and SLO conditions were similar to those reported in muscle of wild rainbow trout from Dospat Dam Lake (Smolyan region, Bulgarie) (Stancheva and Merdzhanova, 2011), this species being naturally richer in n-3 LC-PUFA as compared to cyprinids such as black carp *Mylopharyngodon piceus* and grass carp *Ctenopharyngodon idella* (Hong et al., 2014).

Paulino et al. (2018) observed on juvenile tambaqui that the fish EPA and DHA contents decreased with an increase of the dietary LA/ALA ratio. In the present study, we also observed that the EPA and DHA contents in muscle, as well as in liver, were lower in SFO-fed fish, which presented the highest LA/ALA ratio. Moreover, the muscle of fish fed LO and CLO diets showed the highest n-3/n-6 ratio (1.6 and 1.0 in LO- and CLO-fed fish, respectively) and these n-3/n-6 ratios were higher than those reported in common carp by Stancheva and Merdzhanova (2011); Mráz et al. (2012); and Hong et al. (2014). The dietary n-3/n-6 ratios are implicated in controlling markers of the metabolic syndrome, including insulin sensitivity, inflammation, lipid profiles and adiposity (Burghardt et al., 2010). According to different authors (Bhardwaj et al., 2016; Gómez Candela et al., 2011; Simopoulos, 1991), humans have been evolutionary adapted to a diet with a n-3/n-6 ratio close to 1. Such n-3/n-6 ratio was observed in the muscle of carp fed on the CLO diet but also the LO diet. This observation supports the suitability of linseed oil as plant-derived oil substituting fish oil in carp feeding, not only in terms of carp culture performance, but also from a human nutrition perspective.

4. Influence of plant oil-based diets on immune modulation of common carp

4.1. Influence of plant oil-based diets on immune parameters

4.1.1. In basal conditions

Generally, the overall immune status of common carp was not altered by plant oil utilization in normal conditions even if a reduction of alternative complement activity was recorded in

SO-fed fish (Experiment 3). Similar results were demonstrated in previous studies in Nile tilapia *Oreochromis niloticus* (Ferreira et al., 2015; Larbi Ayisi et al., 2018; Yildirim-Aksoy et al., 2007); black carp (Sun et al., 2011), and Eurasian perch *Perca fluviatilis* (Geay et al., 2015a). On the other hand, the highest level of lysozyme activity was observed in LO fish (Experiment 3) indicating the positive effect of plant oil utilization on this immune parameter. Some authors reported that the diets rich in ALA could improve immune competence and disease resistance (Chen et al., 2016; Cornet et al., 2018; Geay et al., 2015b) ; moreover, the balance between n-3 and n-6 LC-PUFAs might create more favorable immune response and the dietary n-3/n-6 ratio should be close to 1 (Bhardwaj et al., 2016; Gómez Candela et al., 2011; Simopoulos, 1991). In our study, the n-3/n-6 ratio in muscle was found to be close to 1 in linseed oil-based diets (Experiment 1). The latter information could explain why, in our study, alternative complement activity in CLO and LO-fed fish was higher than in SO-fed fish (Experiment 3), and lysozyme activity in LO-fed fish was higher than in SO-fed fish. In the case of supplementation with an immunostimulant (β-glucan), the lower values of lysozyme activity were observed in plant oil-based groups (LO+ and SFO+) compared to fish oil one (CLO+) indicating that the plant oil source in our experiment altered the immunostimulatory action of β-glucan. However, the lowest value of lysozyme activity in our experiment (33.5 U/mL in SFO+) was higher than those reported in other previous studies in the same species (Lin et al., 2012, 2011). In fact, the interaction of nutrition and immunity in fish has long been known, but this relationship is far more complex than originally considered (Siwicki et al., 2006). Previous studies on this aspect have shown the influence of dietary lipid sources on the immune response in fish due to the dietary FA profiles, but information on the effects of individual FAs is still limited (Kiron, 2012). The LC-PUFAs are worked as the mediators in pro-anti-inflammatory response, more frequent, they are the precursors of some active molecules called eicosanoids. These molecules exist as normal physiological products; however, the excess of eicosanoid metabolism occur in the extreme stress condition or other stimulations that trigger the release of phospholipase in the cell membrane phospholipid of these molecules generally link to the chronic inflammatory diseases (Calder, 2017, Chandrasekharan and Sharma-Wali, 2015; Chiurchiu et al., 2018; Medzhitov, 2008; Mullen et al., 2010; Stella et al., 2018; Wall et al., 2010).

4.1.2. In stimulated conditions

In challenge test (experiment 2), the lysozyme activity of SFO-fed fish was comparable with CLO and LO ones but this parameter was lower in SFO+ group compared to CLO+ and LO+ ones. This result indicates that a diet rich in LA induced some alterations in the immunostimulation of β-glucan. However, this could be explained by the anti-inflammatory effect induced by the high level of ARA in SFO-fed fish. ARA is the major precursor of highly active eicosanoids (Bell and Sargent, 2003; Wall et al., 2010) that play a role in immune and inflammatory responses (Sargent et al., 2002; Wall et al., 2010), but this LC-PUFA molecule is also the precursor of lipoxin metabolism (Chiurchiu et al., 2018). Therefore, the lysozyme level in SFO+ group was comparable with CLO-fed fish but lower than CLO+ and LO+ ones.

In LPS-stimulation, the highest value of peroxidase activity was observed in SLO HKL (147.7 U/mL) and other groups were similar (experiment 3). This could be explained by the abundance of both ARA and EPA and more balanced in precursor PUFAs of this experimental oil. In SLO fish, ARA level was higher than CLO and LO group while EPA

level was higher than SO fish; besides, the LA and ALA levels in SLO diet or fish displayed the intermediate values compared to LO and SO groups. The eicosanoids include prostaglandins and leukotrienes (produced from ARA, EPA) and are one of the main pro-inflammatory mediators (Sargent et al., 2002; Wall et al., 2010). High levels of prostaglandin or related gene expression have been reported in fish fed dietary lipid sources rich in LA or ARA (Asturiano et al., 2000; Bell et al., 1993; Tian et al., 2016). Therefore, the highest value of peroxidase activity obtained in SLO group could be explained by these arguments. In experiment 4, the results showed that the NO activity and total Ig were stimulated by LPS in HKL isolated from fish fed plant oil-based diets and these values did not differ with those of CLO HKLs indicating that the selected plant oils induced a good immunocompetence in carp.

4.2. Influence of plant oil-based diets on the expression of genes involved in immune responses

In this current study, we assessed the influence of plant oil utilization as well as the LC-PUFA amounts in fish diet on the expression of several important genes involved in the innate (*lys, b/c2*) and adaptive (*nkef*) immune responses; pro-inflammatory processes (*nf-kb, inos, il-1, il-6, il-8, tnf-α, cxc*); pattern recognition (*tlr-4*); eicosanoid metabolism processes (*pla2, pge2, 5-lox*); anti-inflammatory responses (*il-10, tgf-β, nf-kbi*) and cytoprotective processes (*prdx-3, gpx-1*).

Generally, in the condition without stimulation by bacterial or other exogenous agents, no significant differences were found for these genes between plant oil-fed fish and fish oil-fed fish, indicating that the plant oil utilization did not induce any negative effect on the overall immune status. In combination of plant oils with an immunostimulant (β-glucan) (experiment 2), we found that the plant oil induced the negative effects on the immunomodulation of this compound when the immunostimulations were observed only in CLO+ fish for *nkef*, *lys* and *il-8*. However, the expression of these genes was comparable with LO+ and SFO+ groups. Moreover, the highest expression of *pla* and *pge* genes, two key genes in the eicosanoid metabolism process, in SFO-fed fish liver was explained by the abundance of ARA in SFO-fed fish. An up-regulation of these genes could have induced the secretion of ARA from liver membrane layers of fish in the SFO group and eicosanoid metabolism activity was higher here than other groups. A similar result was published for large yellow croaker *Larmichthys crocea* (Lin et al., 2012) in testing the kidney macrophages with different ARA doses. The SFO diet exhibited the over-regulation of genes involved in eicosanoid metabolism (*pla2, pge2*) in the condition without stimulation that may induce some alterations in fish immune system. However, other candidate plant oils were comparable with CLO diet indicating that the plant oil utilization generally did not induce negative effects on the immune response of this species in the normal conditions. The *pla* and *pge* expression in SFO+ was lower than SFO-fed fish, indicating the immunomodulatory effect of β-glucan in the diet, which was able to inhibit some inflammatory responses such as prostaglandin production and pain response.

In LPS-stimulated condition (experiments 3 and 4), almost all target pro-anti-inflammatory genes assayed were stimulated, including cytokines (*il-1, il-6, tnf-α*), chemokines (*il-8, cxc*), eicosanoids (*pge2*), anti-inflammatory mediators (*il-10, nf-fbi*) and other mediator (*inos*). The inflammatory response plays a crucial role in animal immune system against several injuries or microbial infections (Abdulkhaleq et al., 2018; Chiurchiu et al., 2018; Medzhitov, 2008; Taams, 2018) inducing the up-regulation of these genes in HKL exposed to LPS. However, the expression of these genes in our study varied depending on the dietary oil sources. Besides, we

also observed the time dependence of pro-anti-inflammatory gene expressions. Indeed, we did not observe the LPS-stimulation of HKL after 24h for candidate pro-inflammatory genes (*cxc, il-8* in experiment 3 and *nf-kb, inos, il-1, il-8, cxc, il-6, tnf-α* in experiment 4) while this one was strongly displayed in almost all genes after 4h of LPS exposure. On the other hand, the *pge2* gene expressed the up-regulation in HKL exposed to LPS after 24h. The *cxc* and *il8* are cytokines that can activate eicosanoid production (Dudzinski and Serhan, 2004) and cytokines/chemokine genes are normally expressed at the early stage of the inflammatory processes (< 12 h) (Gonzalez et al., 2007; Wu et al., 2012). These arguments could explain the up-regulation of *pge2* after 24h instead of 4h as other pro-inflammatory genes. Interestingly, expression of anti-inflammatory genes (*il-10, nf-kbi* and *tgf-β1*) also presented an up-regulation at the early stage (4h). This response suggests that both pro- and anti-inflammatory processes have been stimulated simultaneously in order to better balance the immune defense homeostasis as previously reported by Rebl and Goldammer (2018).

We observed an up-regulation of *pge2* expression in HKL isolated from fish fed a diet rich in LA (SLO and SO fish) and also in ALA (LO fish), suggesting that in the condition stimulated by the antigen (or immunostimulant) the HKL prioritised the biosynthesis of ARA, the precursor of eicosanoids in the inflammatory response. However, the expression of this gene in experiment 4 concerned the CLO-fed HKL instead of SLO ones. This discrepancy could be explained by the expression peak of this gene that was not similar at the same sampling. Regarding pro-inflammatory cytokine/chemokine expression, we found that the highest regulation was always observed in CLO HKL exposed to LPS and SLO or dietary plant oils supplemented with ARA (LOA) or DHA (SOD). The lipid mediators play in parallel two roles in the inflammatory response, as pro- and anti-inflammatory actors. At the peak of acute inflammation, very similar cells involved in the production of pro-inflammatory lipid mediators undergo a class switch and start producing specialized pro-resolving mediators (resolvins of D series from DHA, E series from EPA and lipoxin from ARA) by the same enzymes engaged in classical eicosanoid production (Chiurchiu et al., 2018; Serhan, 2014). A higher stimulation was found in groups balanced in lipid mediators (namely CLO, SLO, LOA and SOD groups) in comparison to LO and SO groups. In experiment 1, the ARA levels in fish fed SO were very high (3.8mg/g) and much higher than in fish fed CLO-based diet (0.85mg/g). Consequently, in the case of SO group, the lipoxin (anti-inflammatory mediator produced from ARA) was perhaps synthesized in the same stage and it reduced the ARA-precursor eicosanoid level. This one may conduct to the lower expression of pro-inflammatory cytokine/chemokine even if muscle of fish fed SO diet were rich in ARA. During the inflammation processes, prostaglandin signaling – especially the one mediated by PGE2 and PGI2 – seems to be involved in the sustained inflammation that causes the transition to chronic inflammation by acting as "cytokine amplifiers" (Aoki et al., 2008) conducting to some damages in the animal immune system. Regarding our results, no increases of inflammatory cytokines/chemokine after 24h of culture were recorded, suggesting that plant oils did not induce any negative response during the inflammatory process.

The highest expression level of the anti-inflammatory cytokine gene *il-10* was observed in SOD group and comparable to the one observed in fish fed CLO, SLO and SO at 4h and similar with results of pro-inflammatory cytokine/chemokine expression indicating that anti-inflammatory response had presented in the same period with pro-inflammatory response to

reduce and avoid the damage effect of this process (Rebl and Goldammer, 2018). We found that the expression of *il-10* in LO HKL exposed to LPS was comparable to other groups while the pro-inflammatory cytokine/chemokine expression was down-regulated, suggesting that an anti-inflammatory cytokine, *il-10* regulated the inflammatory process in case of the absence of other anti-inflammatory lipid mediators. Results reported in experiment 2 have shown that even if fish fed LO diet (rich in ALA, DHA – precursor) but the DHA level in tissue was very low compared to CLO ones. Another anti-inflammatory gene, *tgf-β1*, the transforming growth factor, is a secreted ligand that has been intimately linked to the regulation of tumor initiation, progression and metastasis (Bierie and Moses, 2011). This factor plays an anti-inflammatory role in the inflammation (Jin et al., 2014; Sanjabi et al., 2009) and its activity increases with resolvin D1 produced from DHA (Luo et al., 2016). This may explain why the expression of this gene was the highest in SOD group, after both 4 and 24 h of LPS exposure.

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Chapter 9

Conclusions and perspectives

The results obtained from our four experiments confirmed all five hypotheses and fulfilled our objectives.

Conclusions

First we conclude that common carp are able to use plant oil-based diets without any negative effect on growth, feed utilization, survival, and fish resistance. Moreover, the blend of terrestrial vegetable oils rich in LA (sesame oil) and ALA (linseed oil) induced a higher feed utilization than pure plant oil- and even than fish oil-fed fish.

The tissue FA compositions reflect those of the lipid sources. Moreover, common carp are able to convert the LC-PUFAs from PUFA precursors. Fish fed LA-enriched oil benefited from higher ARA levels than fish oil-fed fish while higher levels of EPA were found in ALA-enriched groups compared to LA-enriched ones. The mixture of LA and ALA-enriched plant oils provided an oil source more balanced in PUFA precursors, allowing a more balanced LC-PUFA profile in fish tissues (liver and muscle) compared to those observed in pure plant oil-fed fish.

The deficiency of LC-PUFAs in fish fed plant oil-based diets induced some negative effects on immune responses in common carp but resistance to disease was not affected. LPS clearly stimulated head kidney leucocytes (not peripheral blood mononuclear cells) in fish, and immune variables as well as expression of genes involved in innate immune system, inflammatory responses, and eicosanoid metabolism processes were modified according to the dietary lipid sources. The diet that was more balanced in FA composition by using a mixture of two plant oils or a supplementation of LC-PUFA to pure plant oils induced higher immune responses than pure plant oil sources, and the results were comparable to those observed in fish oil-fed fish.

The *in vitro* combined with *in vivo* approaches helped to observe a remarkable influence of lipid sources on the fish immune responses via the assessment of indicators including humoral immune variables and key genes involved in such processes in key cells.

Perspectives

Plant-derived oils should be encouraged to be applied in aquaculture feed production. Research should be extended to other species using these lipid sources instead of fish oil in aquatic feed industry, especially in marine fish culture. Further, the combination of several terrestrial vegetable oils is recommended to provide a more balanced PUFA profile for fish species that are able to convert LC-PUFAs from PUFA precursors.

The advantages of bioengineer plant oils enriched in LC-PUFAs including economical and nutritional aspects were demonstrated. These lipid sources do not contain any genetic material (nucleotide and protein). However, research to confirm their safety for human and animal health should be investigated.

We observed the influence of ALA/LA as well as n-3/n-6 PUFA ratios on the immune system in common carp. We recommend further research to determine the optimal values of these ratios that could be provided from different mixture rates of plant oils. Moreover, in fish diet, beside of fatty acids, other ingredients such as amino acids, vitamins, and minerals can also influence the fish immune system. Therefore, studies focusing on the interaction between fatty

acids and other nutrients on the fish immune responses should be conducted. The obtained results maybe support for optimizing diet formulation in fish.

In the current study, we have determined the influence of dietary lipid sources on fish immune system by the assessment of humoral immune variables and expression of genes involved in immune responses as well as the interaction between lipid nutrition and immunology. However, beside of this research, other methodologies such as proteomics, as well as the measurements of some target proteins (prostaglandin, lipoxin, or resolvin) are also recommended in future studies to provide a more complete picture of these interactions. Furthermore, the lipid nutrition may affect the intestine health and bacterial populations that also plays an important role in fish immune system. Therefore, studies investigating the influence of plant oil utilization instead of fish oil on intestine health as well as on intestinal microbiota are susceptible to provide relevant results in order to better understand these complex interactions between lipid nutrition, immunity and health in fish.

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Publications

Journal publications

Thi Mai Nguyen, Syaghalirwa N.M. Mandiki and Patrick Kestemont (In preparation) Innate immune and pro-anti-inflammatory responses of common carp *Cyprinus carpio* as modified by dietary plant oils and a DHA or ARA supplementation in these oils after LPS injection

Research fields

Aquaculture
Biotechnology applied in Aquaculture
Fish immunology
Fish nutrition

Thi Mai Nguyen, Syaghalirwa N.M. Mandiki and Patrick Kestemont (In preparation) Review: Updates of the influence of dietary fish oil substitution by plant oils on fish fatty acid composition and immune responses

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Conference posters

Thi Mai Nguyen, Thi Nang Thu Tran, Yvan Larondelle, Robert Mandiki, Patrick Kestemont (2018) Beneficial combination of β–glucan with different dietary lipid sources on growth, immune response, fatty acid profile and expression of several genes involved in immunology, lipid biosynthesis and eicosanoid process in common carp (*Cyprinus carpio*) International symposium on fish nutrition and feeding (ISFNF2018) in Las Palmas de Gran Canaria, Spain

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